UNIVERSIDADE DE LISBOA

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Acknowledgements

To Professor Ana Duarte, for the enthusiasm she has shown receiving me and guiding me. For all the good times, joy and optimist, even in the hardest moments.

To Professor José Meireles, for having accepted to co-supervise me and for all the help and availability.

To Dra. Regina Hofmann-Lehmann, and her team, for making this work possible

To Dra. Lídia Gomes, for all the patience and help, but especially for the constant good mood at the lab, which has helped me pull through one of the most difficult stages of the work.

To Professor Telmo Nunes, for his availability and invaluable help with the statistical analysis.

To the entire team at the clinic MyVet, Dr. Nuno, Márcia, Dra. Anna and Patrícia, for the way they have received me, for having believed in me and for all the patience, advice and knowledge that have immeasurably contributed to my professional and personal growth.

To Dra. Marta Videira and the entire team at Casa dos Animais, for having welcomed me and helped me in everything I needed.

To Joana, for all the companionship throughout this complicated journey. All the best moments I remember in this journey are marked by your presence and Ana's. Thank you for your friendship and for being there whenever I need.

To my partner, for the love and unconditional support. For having helped me get up everytime I've fallen during these years and for never letting me give up.

To my entire family, who have always helped me and allowed all of this to happen.

Abstract

FELINE HEMOPLASMAS: EVALUATION OF SPECIFIC ANTIBODIES AND THE MOLECULAR AND CYTOLOGICAL DIAGNOSTIC

Mycoplasma haemofelis, Candidatus *Mycoplasma haemominutum* and Candidatus *Mycoplasma turicensis* are three hemoplasmas responsible for feline hemolytic anemias. The diagnosis of the infection can be performed by observation of the agents on blood smears or by their identification and quantification using PCR techniques, presently considered the gold standard method. A recombinant antigen for *Mhf* (rDnaK) has been identified, characterized, produced and then successfully applied in Western blot and ELISA assays (rELISA) to detect antibodies in samples from experimentally induced infections. To evaluate this application under field conditions, this rELISA was used in samples collected from naturally infected cats, in parallel with quantitative PCR and cytologic examination. Within the scope of the TNR program implemented by the Lisbon City Council, blood samples were collected from 104 cats. After sample collection, blood was immediately used to prepare blood smears which were then stained with Giemsa (Parasitology Lab, FMV/Lisbon University). The remaining blood was split into two aliquots: 1 – Plasma separation, shipped to Vetsuisse Faculty, Zurich University, for antibody testing with rELISA; 2 – Total DNA extraction for identification and quantification of the three species of hemoplasma using qPCR (Virology Lab, FMV/Lisbon University). Out of 22.1% (N=23) samples where the microorganism was identified by qPCR, mycoplasmas were also identified on the blood smears of 15.4% (N=16). 4.8% (N=5) of samples tested as seropositive and 2.9% (N=3) revealed bordeline results. From 77.9% (N=81) of samples that were negative by qPCR, only 44.2% (N=46) were also negative on cytologic examination. 60.6% (N=63) of samples were considered seronegative and 6.7% (N=7) were borderline. Sensitivity for cytology was 69.6% and specificity was 56.8%. Sensibility of rELISA was 25% and its specificity was 85.1%. Cohen's Kappa (k) was calculated to assess agreement between PCR-Cytology (k=0.1836) and PCR-rELISA (k=0.1093). Given the low agreement, PCR was found to be the most appropriate diagnostic method. Further studies are necessary to characterize the response of the immune system and the role of different antigens in these infections in order to improve the suitability of rELISA in a clinical setting

Keywords: *Mycoplasma haemofelis*, Candidatus *Mycoplasma haemominutum*; Candidatus *Mycoplasma turicensis*; Hemoplasmas; quantitative PCR; rELISA

Resumo

HEMOPLASMAS FELINOS: AVALIAÇÃO DE ANTICORPOS ESPECÍFICOS E CORRESPONDÊNCIA COM O DIAGNÓSTICO MOLECULAR E CITOLÓGICO

Mycoplasma haemofelis, Candidatus *Mycoplasma haemominutum* e Candidatus *Mycoplasma turicensis* são três hemoplasmas responsáveis por anemias hemolíticas em felinos. O diagnóstico desta infecção pode ser feito pela observação dos agentes em esfregaços de sangue ou através da sua identificação e quantificação pelas técnicas de PCR, sendo este ultimo considerado o método *gold standard*. Um antigénio recombinante do *Mhf* (rDnaK) foi identificado, caracterizado, produzido e posteriormente aplicado com sucesso, em técnicas de *Western blot* e rELISA, para a deteção de anticorpos em amostras colhidas de gatos infetados experimentalmente. De forma a avaliar a sua aplicação numa realidade clínica, esta rELISA foi utilizada em amostras colhidas de gatos naturalmente infectados, em paralelo com o PCR quantitativo e com o exame citológico. No âmbito do programa CED efetuado pela Câmara Municipal de Lisboa, foi realizada a recolha de sangue a 104 gatos. Após a colheita das amostras, o sangue foi utilizado para preparar esfregaços, corados de seguida com coloração Giemsa (Laboratório de Parasitologia, FMV/ULisboa), e dividido em duas alíquotas: 1- Separação do plasma, que foi enviado para a Faculdade Vetsuisse, Universidade de Zurique, onde realizaram a pesquisa de anticorpos com a rELISA; 2 – Extracção do ADN total para a identificação e quantificação das três espécies de hemoplasmas por qPCR (Laboratório de Virologia, FMV/ULisboa). Das 23 (22.1%) amostras PCR positivas, foram igualmente encontrados micoplasmas em 16 esfregaços (15.4%). 5 (4.8%) amostras foram consideradas seropositivas e 3 (2.9%) apresentaram resultados inconclusivos. Das 81 (77.9%) amostras negativas ao PCR, apenas 46 (44.2%) mostraram-se negativas ao exame citológico. 63 (60.6%) amostras foram consideradas seronegativas e 7 (6.7%) apresentaram valores não conclusivos. A sensibilidade da citologia foi de 69.6% e a especificidade foi de 56.8%. Por sua vez, a rELISA apresentou uma sensibilidade de 25% e uma especificidade de 85.1%. O Cohen's Kappa (k) foi calculado para avaliar a concordância entre o PCR e a Citologia (k=0.1836) e o PCR e a rELISA (k=0.1093). Tendo em conta as baixas concordâncias, o nosso estudo confirma que o PCR é o método de diagnóstico mais adequado. Mais estudos são necessários para caracterizar a resposta imunitária e o papel dos vários antigénios destes agentes, de forma a melhorar a sua aplicação no rELISA e esta poder ser usada no contexto clínico.

Palavras Chave: *Mycoplasma haemofelis*, Candidatus *Mycoplasma haemominutum*; Candidatus *Mycoplasma turicensis*; Hemoplasmas; PCR quantitativo, rELISA

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List of Abbreviations

AI - After infection
BID - Twice daily
CEBEA - Ethics committee for animal welfare
CI - Confidence interval
C*Mhm* - *"*Candidatus *Mycoplasma haemominutum"*
C*Mhp* - *"*Candidatus *Mycoplasma haematoparvum"*
C*Mt* - *"*Candidatus *Mycoplasma turicensis"*
cPCR - Conventional PCR
CPDA-1 - Citrate-phosphate-dextrose adenine
DAI - Day after infection
DNA - Deoxyribonucleic acid
DnaK - Heat shock protein
dNTPs – Deoxynucleotides, used in the PCR reaction
e.g. - For example
EDTA - Ethylenediaminetetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
EM - Electron microscopy
FeLV - Feline leukemia virus
FIV - Feline infectious virus
FMV-UL - Faculty of Veterinary Medicine – University of Lisbon
FWD - Forward
h - Hours
HspA1 - Mycoplasma suis antigen, that belongs to the DnaK family
IgG - Immunoglobulin G
IgM - Immunoglobulin M
IM - Intramuscular
kg - kilogram
mg - Milligram
Mhf - *Mycoplasma haemofelis*
ml - Milliliter
NCBI - National Centre for Biotechnology Information
ng - Nanogram
nm - Nanometre
OD – Optical density
ºC - Degree Celsius
PCR - Polymerase chain reaction
PO - Per os; Oral administration
qPCR - Quantitative polymerase chain reaction
rELISA - Recombinant ELISA
REV - Reverse
RNA - Ribonucleic acid
rnpB - gene RNase P
SID - Once daily
SPF - Specified pathogen-free
TEM - Transmission electron microscopy
TID - Three time daily
TNR program - Trap-Neuter-Return program
µl - Microlitre
µm - Micrometer

1. Curricular training report

Curricular training was performed in three stages: the first at Casa dos Animais de Lisboa (Figure 1) for a period of 4 months (May, June, July and September 2017), supervised by Dr. Marta Antas Fernandes Videira; the second stage was performed in the Virology and Molecular Biology Laboratory as well as the Parasitology and Parasitic Diseases Laboratory of the Faculty of Veterinary Medicine – University of Lisbon (FMV-UL), under the supervision of Doctor Ana Isabel Simões Pereira Duarte and Doctor José Augusto Farraia e Silva Meireles. This phase was undertaken in parallel with the first one; the third and last stage took place in the Veterinary Center MyVet, in Paço de Arcos, and lasted for 6 months. It started in October 2017 and ended in March 2018. It was supervised by Dr. Nuno Miguel de Almeida Monteiro Sousa.

1. Casa dos Animais de Lisboa

On this first stage, sample collection for the proposed experimental work took place as part of the Trap-Neuter-Return (TNR) program run by the Lisbon City Council to control free roaming cats. This stage took place in the mornings, between 10am and 1pm, corresponding to a total of 360h of curricular training. Samples were total blood samples collected from the jugular vein while the animal was under anaesthesia. Collection took place in a total of 104 free roaming cats. On top of the sampling, I was also able to participate in the pre-op preparation of the patients and assist in the surgery.

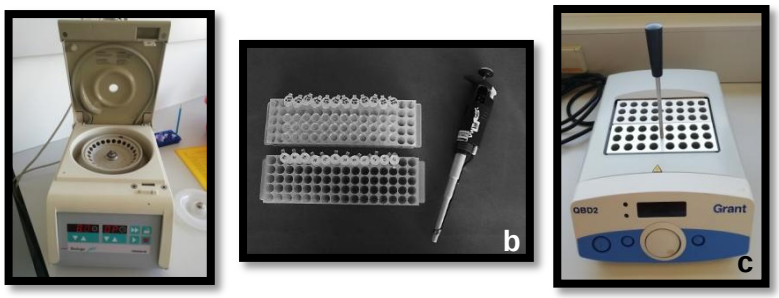
Figure 1. Casa dos Animais de Lisboa.
(Image from: http://www.cm-lisboa.pt/equipamentos/equipamento/info/casa-dos-animais-de-lisboa)

2. Faculty of Veterinary Medicine – University of Lisbon

2.1. Virology and Molecular Biology Laboratory

After collecting blood samples in the morning, they were processed between 3pm and 6-7pm (total 450h). During this stage, on top of separating plasma samples to be sent to Zurich, there was a more thorough understanding and performance of DNA extraction techniques and its use in quantitative PCR (qPCR) protocols, in order to identify feline hemoplasmas (Figure 2).

Figure 2. Photos taken at the laboratory during the procedures: a - centrifuge; b - samples processing; c - block heater



2.2. Parasitology and Parasitic Diseases Laboratory

During this stage, blood smears from the sample stage were stained for observation. In order to do this, I was taught the technique for the fixation and Giemsa staining of the samples (35h). After staining, they were then observed under the microscope (98h).

3. Curricular training in Small Animal Practice

This stage took place at the MyVet Veterinary Center (Figure 3) and lasted for a total of 1440 hours consisting of participation in the different services provided by the clinic.

In internal medicine, several tasks were developed as part of pet consultations, such as obtaining the anamnesis, performing the physical examination, animal restraint, general medicine procedures (e.g. ear cleaning, bandaging), preventive medicine (e.g. vaccination, de-worming), biologic samples collection for complementary diagnostics (e.g. blood sampling, skin scrapes, fine needle aspiration) and medical emergencies. It was also possible to follow different clinical cases, which allowed me to learn about the approach for various diseases, as well as their treatment and follow-up.

Regarding imaging, I assisted with several radiology and ultrasound diagnosis, both with the animal restraint and with the interpretation of results. I also had the opportunity to assist with procedures such as ultrasound-guided sampling and echocardiography.

In the surgical field, I assisted with the pre-op procedures and the anesthetic monitoring before, during and after surgery.

When working with inpatients, I undertook varied tasks such as placing intravenous catheters, monitor patients, feed them and insure they were kept in clean conditions.

Figure 3. MyVet – Veterinary Center.

4. Presentation of results

This work was accepted as a poster communication on the CIISA congress, on 16th to 17th November 2018, in Portugal, with the title: “Feline hemoplasmas: evaluation of specific antibodies and the molecular and cytological diagnostic” (Appendix A).

2. Bibliographic review

2.1 Etiology

Hemotropic mycoplasmas, also known as hemoplasmas, are epicellular, pleomorphic, gram-negative cells that don't possess a cell wall and attach to the surface of erythrocytes. They are responsible for clinical presentations of infectious hemolytic anemia in a wide range of mammal species (Rikihisa et al., 1997; Sykes, 2010a; Messick & Harvey, 2011).

The observation of hemoplasmas was first documented by Clark (1942), when he observed structures with a similar morphology to *Eperythrozoon* genus on the blood smear of an anemic cat in Pietermaritzburg, South Africa, proposing to classify them as *Eperythrozoon felis*. In 1953, similar organisms were found in a cat in Colorado, United States of America, and their presence was associated with episodes of moderate to severe hemolytic anemia, resulting in a disease designated as Feline Infectious Anemia (Flint & Moss, 1953). In 1955, taking into consideration their morphology, Flint and McKelvie propose to classify these organisms as *Haemobartonella felis* (*H. felis*) (Sykes, 2010a).

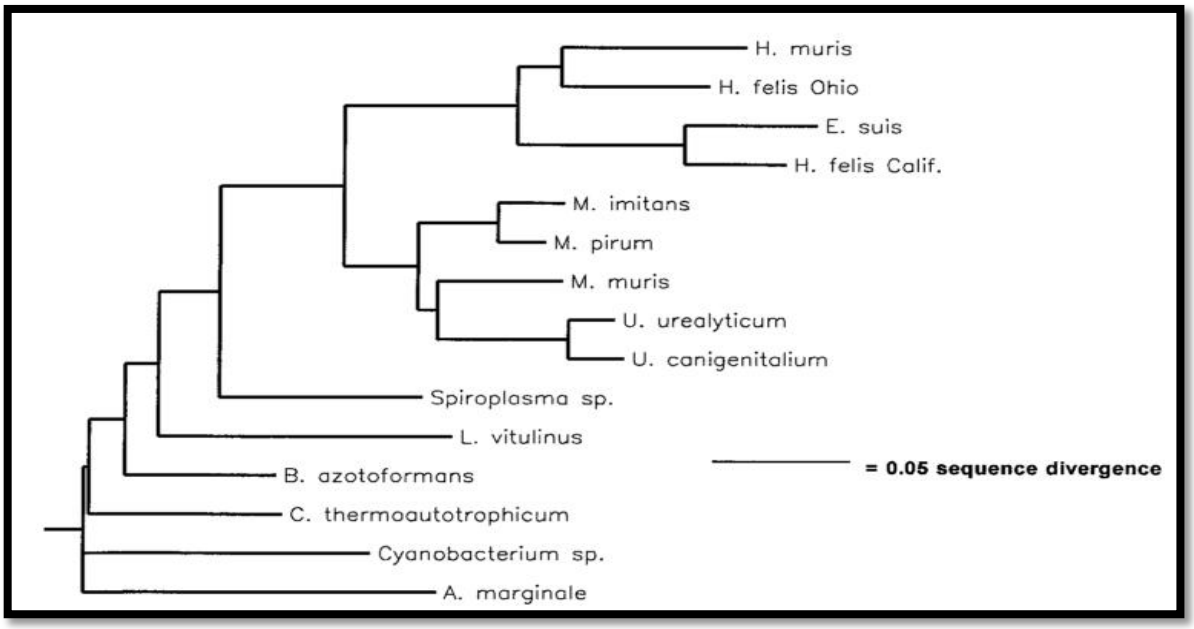
In this first stage, genera *Haemobartonella* and *Eperythrozoon* were placed within the order *Rickettsiales* based on their small size (<1.0 µm), their staining properties (gram-negative), possible transmission via vectors (hematophagous arthropods), the inability to culture them in vitro and their hemotrophic characteristics (family *Anaplasmataceae*) (Neimark, Johansson, Rikihisa & Tully, 2001; Willi et al., 2007a). At this point, the individual classification of each species was performed based on the host where they were identified, since it was believed that most of these microorganisms were species-specific. (Neimark et al., 2001). The distinction between the two genera was based on the microorganisms morphology and localization. Species belonging to the genus *Eperythrozoon* frequently present a ring shape and can be found both inside erythrocytes as well as free on the plasma, while species belonging to the genus *Haemobartonella* are attached to the erythrocytes, rarely being found on the plasma, and the ring shape is seldom, if ever, detected (Rikihisa et al., 1997; Neimark et al., 2001).

However, unlike other bacteria belonging to the order *Rickettsiales*, these organisms don't multiply inside the cells, simply adhering to erythrocyte membranes, and don't have a cell wall (Neimark & Kocan, 1997; Rikihisa et al., 1997). Because it was not possible to culture them in a laboratory setting, more comprehensive research was not achievable, something that only changed in the 90's with the development of PCR techniques (Willi et al., 2007a; Sykes, 2010a). With the amplification, sequencing and phylogenetic analysis of the gene 16S, which codes a subunit of ribosomal RNA, new information was obtained regarding the phylogenetic proximity between the different known species of the genera *Haemobartonella* and *Eperythrozoon*, as well as their relation to species belonging to the genus *Mycoplasma* and the family *Anaplasmataceae* (Figure 4) (Rikihisa et al., 1997; Foley, Harrus, Poland,

Chomel & Pedersen, 1998). This analysis was performed in various blood samples obtained from cats infected with hemobartonellosis and the conclusion was that there are at least two strains of *H. felis*: the California strain (small variant, with about half the size of the Ohio strain), and the Ohio strain (large variant), which causes more severe clinical cases and is, therefore, more pathogenic (Rikihisa et al., 1997; Foley et al., 1998). They were also observed to have close proximity with species belonging to the genus *Mycoplasma*, such as *Mycoplasma fastidiosum*, isolated from the respiratory tract of horses, and *Mycoplasma cavipharyngis*, isolated from the nasopharynx of guinea pigs. The genetic similarity suggested their inclusion on the genus *Mycoplasma* and not the order *Rickettsiales,* in which they had initially been classified based on their morphologic and biologic characteristics (Rikihisa et al., 1997; Foley et al., 1998; Johansson, Tully, Bölske & Pettersson, 1999). On top of molecular similarities, these organisms share other characteristics, such as: small size, absence of a cell wall or flagella, demanding culture conditions, sensitivity to tetracyclines and resistance to penicillins (Neimark et al., 2001; Willi et al., 2007a; Tasker, 2010).

These organisms were therefore included in the genus *Mycoplasma*, with the proposal of being designated as "hemoplasmas" to characterize and differentiate this microorganism group with hemotropic abilities (Neimark et al., 2001). The change affected the entire genus *Haemobartonella* and the majority of the organisms in the genus *Eperythrozoon* (Johansson et al., 1999; Willi et al., 2007a). The Ohio strain was designated *Mycoplasma haemofelis* (*Mhf*) (Neimark, Johansson, Rikihisa & Tully, 2002) and the California strain was designated *"*Candidatus *Mycoplasma haemominutum"* (C*Mhm*).

Figure 4. Phylogenetic relation between *Haemobartonella* spp., *Mycoplasma* spp and other species of bacteria (Rikihisa et al., 1997).



The taxonomic prefix “Candidatus” reflects the temporary nature of this classification, since the inability to culture them in vitro does not allow for a proper characterization, neither of their phenotype or genotype (Neimark et al., 2001, 2002; Willi et al., 2007a).

However, this new classification led to some controversy. There is general consensus that the phylogenetic analysis of the 16S rRNA gene sequence resulted in enough evidence to support the transfer of hemoplasmas into the family *Mycoplasmataceae*. However, the same doesn’t apply to their inclusion in the genus *Mycoplasma*. The small size of the used sequence and the fact that the genetic similarities between the species of both genus was inferior to that of other species in well studied genera, such as the similarities between *Staphylococcus* and *Streptococcus*, *Clostridium* and *Bacillus* or *Corynebacterium* and *Mycobacterium*, demonstrates that hemoplasmas should be a new genus inside the *Mycoplasmataceae* family. Another argument that supports this relates to the biologic differences found: mycoplasmas colonize the respiratory and urogenital mucosa, rarely penetrating to the level of the submucosa and going into circulation; several mycoplasmas can be cultured, despite needing complex growth mediums; the means of transmission may also be different (Rikihisa et al., 1997; Uilenberg, Thiaucourt & Jongejan, 2004; Messick & Harvey, 2011).

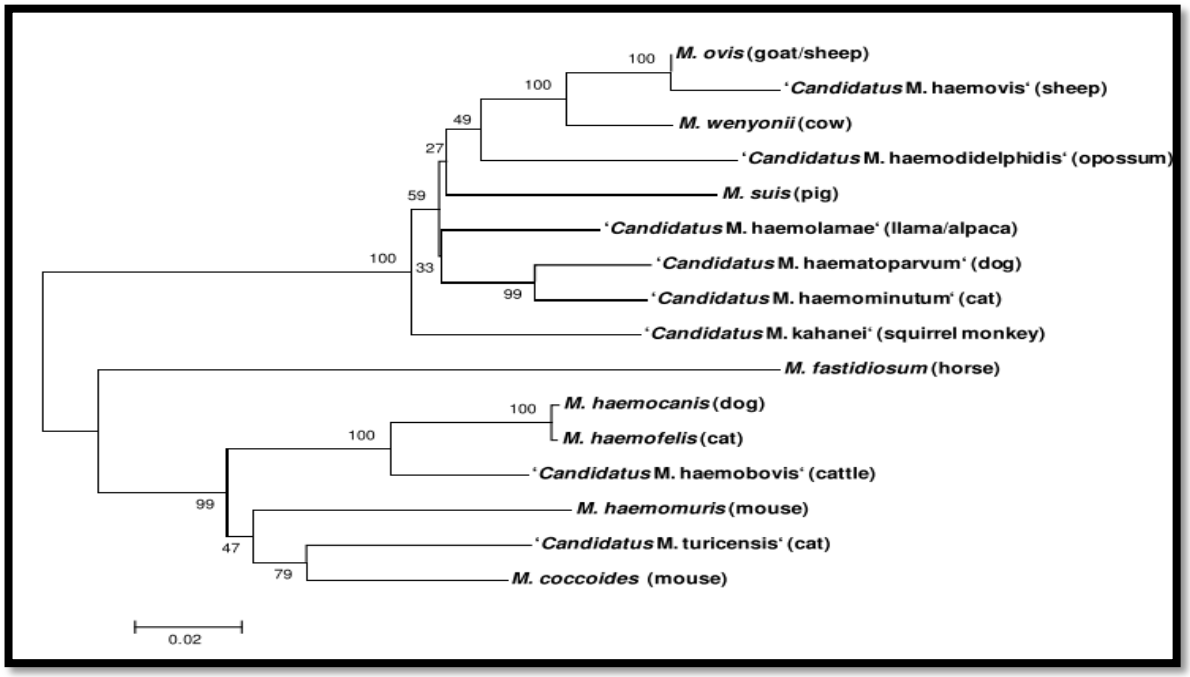
In an epidemiologic study that had the objective of determining the prevalence of hemoplasma infections in cats in Switzerland, a new species was isolated, which was designated as *“*Candidatus *Mycoplasma turicensis”* (C*Mt*) (Willi et al., 2005, 2006b). This variant was seen to have very close phylogenetic proximity with the species *Mycoplasma haemomuris* and *Mycoplasma coccoides*, usually found in rodents. It was then speculated if inter-species transmission via predation may have occurred (Willi et al., 2005, 2006a).

It was initially thought that hemoplasmas within the genus *Mycoplasma* belonged to the group pneumoniae, but the analysis and sequencing of gene RNase P (*rnpB*), which codes the RNA subunit of endoribonuclease P, suggested that hemoplasmas are a separate group, despite phylogenetic proximity indicating that there is a common ancestor. This group is then divided in two branches based on their genetic proximity: the haemofelis group branch, with *Mhf* and *CMt*, and the haemosuis group branch, with *CMhm* (Peters et al., 2008a). The conclusion of this work is similar to that found in other publications, in which the analysis of gene 16S rRNA revealed that, despite the phylogenetic proximity with species inside the group pneumoniae, there were divergences in some portions of the genetic sequencing. It was demonstrated that the members of this group and hemoplasmas only have 18 of 30 nucleotides in common (Johansson et al., 1999; Messick, Walker, Raphael, Berent, & Shi, 2002).

These studies allowed the assessment of proximity between different hemoplasmas, as well as their relation to other species of mycoplasmas (Figure 5). These molecular techniques are fundamental, since it’s not possible to use phenotypic characteristics for their

characterization. The sequencing of gene *rnpB* appeared to be more specific to compare close species and strains, since it's present in every bacteria and presents lower variety among individuals (Fox, Wisotzkey & Jurtshuk, 1992; Nübel et al., 1996; Täpp, Thollesson & Herrmann, 2003). These results suggested, once again, a close proximity between hemoplasmas and mycoplasmas, supporting their classification within the same genus (Tasker et al., 2003a; Peters et al., 2008a).

Figure 5. Phylogenetic tree of the most common hemoplasmas. Hosts are indicated in brackets (Willi et al., 2010).



A new species of mycoplasma was identified in a dog with lymphoproliferative neoplasia (leukemia) that had undergone a splenectomy (Sykes et al., 2004), being denominated "Candidatus *Mycoplasma haematoparvum"* (C*Mhp*) (Sykes, Ball, Bailiff, & Fry, 2005). Its morphologic characteristics and phylogenetic analysis via sequencing of genes 16S rRNA and *rnpB* demonstrated very close proximity with *CMhm*, which has interestingly been associated with rapid development of hematopoietic neoplasia in cats infected with the feline leukemia virus (FeLV) (Sykes et al., 2004; 2005). Another hemoplasma genetically close to *CMhp* was identified in California cats, raising the possibility of a fourth species associated with this host (Sykes, Drazenovich, Ball & Leutenegger, 2007b).

Up to this date, in vitro culture of hemoplasmas has been unsuccessful, making it difficult to obtain large quantities of purified DNA (Barker et al., 2011b). Samples must be initially collected from an infected host, resulting in contamination with their own cells. It is, therefore, only possible to obtain small amounts of the genome, making the study of these bacteria more challenging (Barker et al., 2011b; Barker & Tasker, 2013). With the development of

sequencing techniques, it has been possible to isolate the entire genomic sequence of both *Mhf* (Barker et al., 2011a) and *CMhm* (Barker et al., 2012). This breakthrough opened the doors to a deeper understanding of the biochemical processes used by these bacteria, which is important when it comes to developing a culture medium appropriate for their growth in a laboratory setting and performing proteomic and immuno-proteomic studies with the ultimate goal of identifying pathogenicity factors, evasion mechanisms to the host immune system and establishment of the carrier status (Barker et al., 2011b; Santos et al., 2011; Barker & Tasker, 2013).

2.2 Morphology

Examination of feline hemoplasmas with optic and electron microscopy (EM) has been helpful for the morphologic characterization of these microorganisms. With the latter technique, their epicellular position has been demonstrated, as well as the presence of a high level of pleomorphism, with the microorganisms presenting with the shape of a rod or as a donut, discoid, coccoid or conic shapes (Figure 6 and 7). They are found in depressions along the surface of erythrocytes and between that and the microorganism there is a space of 15-25nm in which fibrils connecting to the erythrocyte membrane can be observed. Infected erythrocytes are subject to morphologic changes, losing their typical biconcave shape. Areas where the microorganisms have been attached present depressions and circumscribed erosion lesions (Figure 8). A single membrane involves these organisms, and although no cytoplasmatic organelles have been recognized, several granules with different sizes and densities have been detected. Obtained pictures also suggest that there may two types of cell division possible: binary fission and budding (Figure 6) (Demaree & Nessmith, 1972; Jain & Keeton, 1973; Yang, Yuan, Yu & Hua, 2007; Messick & Harvey, 2011).

In other publications, it's been observed that the three species have very similar morphology, differing only when it comes to their size: *Mhf* has a diameter of 0.5µm, while C*Mhm* and C*Mt* have a smaller size at 0.3µm (Figure 6 and 7) (Willi et al., 2011). In this particular study, transmission electron microscopy (TEM) was used and C*Mt* was seen to have an oval shape, a size of 0.25 µm and had several electron-dense structures inside (Figure 7).

Figure 6. Visualization of *Mhf* (a) and *CMhm* (b) by EM. Discoid organisms can be seen, with about 0.6µm (a) and 0.3µm (b), attached to the surface of erythrocytes. Arrow represents an organism in binary fission (a); bar represents 1µm (Willi et al., 2011).

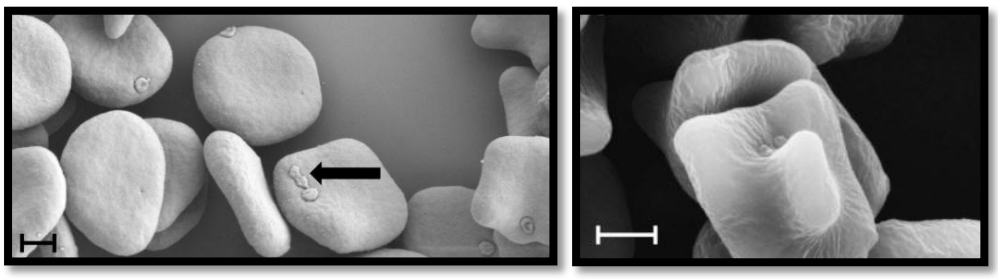


Figure 7. Visualization of *Mhf* (a) and C*Mt* (b) by TEM; (a) – *Mhf* is rod-shaped and has a diameter of 0.5-0.6 µm; (b) – Oval structure with 0.25µm and electrodense material inside; bar represents 0.5µm (a) and 0.2µm (b) (Willi et al., 2011).

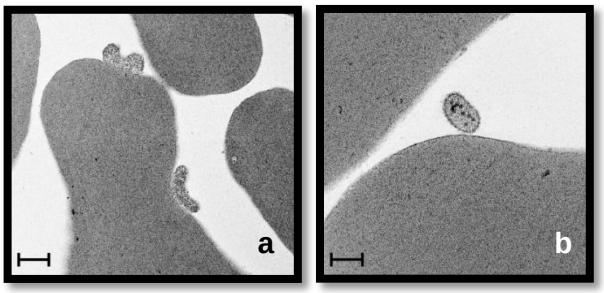
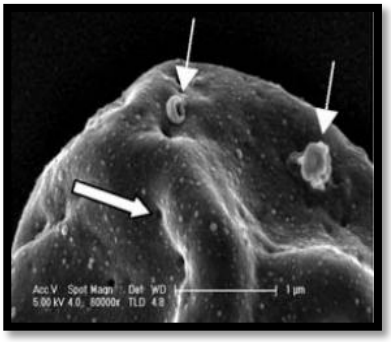


Figure 8. Visualization of hemoplasmas attached to the surface or erythrocytes (arrows); areas of depresssions can also be seen where hemoplasmas have been previously attached (thick arrow) (Yang et al., 2007).



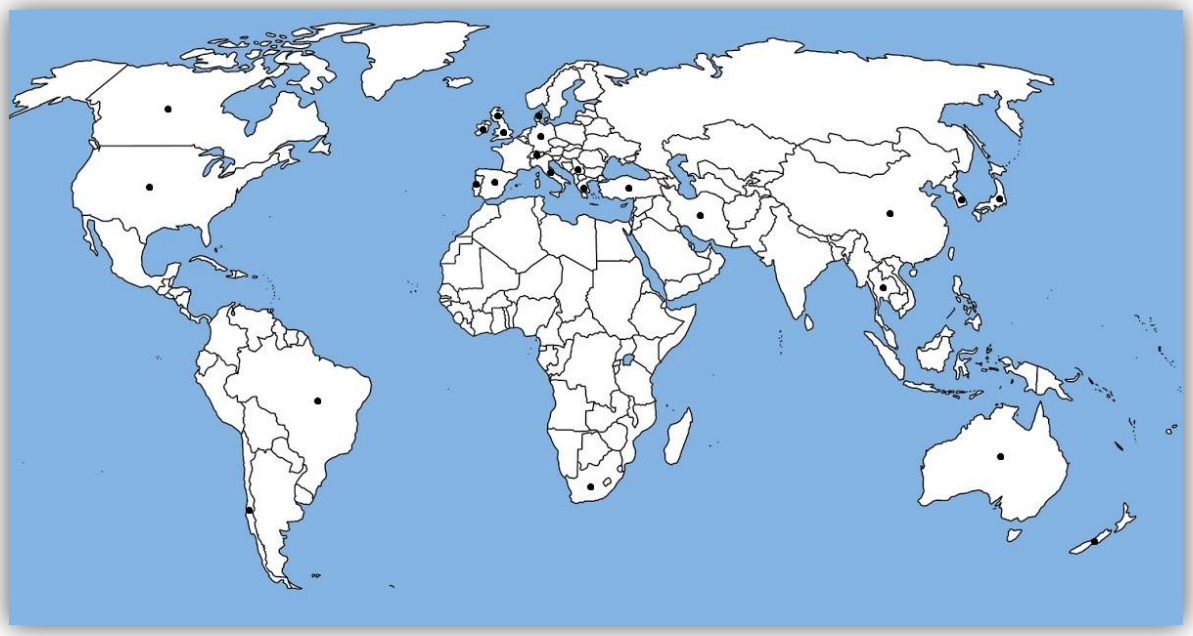
2.3 Prevalence and risk factors

There is limited value in comparing available studies evaluating the prevalence of hemoplasmas in different countries, attributed to: geographic variations, different diagnostic techniques used and statistical treatment of data, size and characteristics of samples and inclusion criteria. Used samples are diverse and may include owned cats with or without outdoor access, stray cats, which may be in shelters, or feral cats. Inclusion criteria are also diverse, from cats characterized as anemic, sick, healthy or even with an unknown clinical status. Other factors that may greatly affect results are geographic location and climate. Locations with higher temperatures are associated with higher prevalence, and this is also verified in areas where several risk factors coalesce (Bauer, Balzer, Thüre & Moritz, 2008; Gentilini et al., 2009; Messick & Harvey, 2011; Martínez-Díaz et al., 2013).

Worldwide prevalence of hemoplasmas is 4-49.4%. The prevalence of *Mhf, CMhm* and *CMt* is 0.5-12.81%, 3.3-41.56% and 0.3-6.7%, respectively (Tasker et al., 2004a; Willi et al., 2006a; 2006c; Yu et al., 2007; Ishak, Radecki & Lappin, 2007; Sykes, Terry, Lindsay & Owens, 2008a; Peters, Helps, Willi, Hofmann-Lehmann & Tasker, 2008b; Kamrani, Parreira, Greenwood & Prescott, 2008; Gentilini et al., 2009; Roura et al., 2010; Maher et al., 2010; Tanahara et al., 2010; Laberke, Just, Pfister & Hartmann, 2010; Stojanovic & Foley, 2011; Bennett, Gunn-Moore, Brewer & Lappin, 2011; Braga, André, Freschi, Teixeira & Machado, 2012; Lobetti & Lappin, 2012; Jenkins, Dittmer, Marshall & Tasker, 2013; Martínez-Díaz et al., 2013; Vergara et al., 2016; Cetinkaya, Haktanir, Arun & Vurusaner, 2016; Rosenqvist et al., 2016; Sarvani et al., 2018). Worldwide hemoplasmas distribution is represented in Figure

9. Of the four identified mycoplasma species, the most prevalent is *CMhm*, followed by *Mhf* and by *CMt*. In Europe, the prevalence of hemoplasmosis varies from 10% to 43.3% with the prevalence of *CMhm* at 5.3-41.6%, *Mhf* at 1.4-12.8% and C*Mt* at 0.3-5.9% (Willi et al., 2006a; 2006c; Peters et al., 2008b; Gentilini et al., 2009; Maher et al., 2010; Roura et al., 2010; Martínez-Díaz et al., 2013; Duarte et al., 2015; Rosenqvist et al., 2016; Sarvani et al., 2018). Known prevalence by district in Portugal (Martínez-Díaz et al., 2013; Duarte et al., 2015) is represented on Figure 10.

Figure 9. Worldwide distribution of hemoplasmas based on available literature (Image adapted from: http://www.santiago.pro.br/mapas/mundo/mundo_mudo_carto.JPG).



Legend: South Africa; Germany; Australia; Brazil; Canada; Chile; China; Korea; Denmark; Scotland; Spain; EUA; Greece; England; Iran; Ireland; Italy; Japan; New Zealand; Portugal; Serbia; Switzerland; Thailand; Turkey.

There is a higher prevalence in males, particularly intact males, likely due to their more aggressive and territorial behavior (Tasker et al., 2004a; Willi et al., 2006c; Gentilini et al., 2009; Bergmann et al., 2017). Age is also associated with the prevalence of hemoplasmosis. Older cats (over the age of 4 years) have been shown in several studies to be diagnosed more frequently with C*Mhm*, while in younger cats (less than 3 years), *Mhf* is more frequently detected. This may be related to the fact that older cats may have had longer exposure times to this agent, having more contact opportunities. This, alongside the fact that *CMhm* has been associated with persistent chronic infections without clinical signs (perhaps because the organism has less ability for elimination than other mycoplasmas), may explain why this mycoplasma has also been associated with males older than 4 years old, with outdoor

access and no clinical symptoms (Grindem, Corbett & Tomkins, 1990; Tasker et al., 2003b; 2004a; Duarte et al., 2015). Around 10% of the healthy feline population will come into contact with mycoplasma agents at some point in their life (Tasker, 2010). On the other hand, a higher susceptibility to *Mhf* in younger cats has been identified, resulting in clinical presentations of anemia. This may be explained by an increased vulnerability of the immune system (Sykes et al. 2008a; Duarte et al., 2015). Outdoor access has been identified as a significant risk factors, both due to the exposure to transmission vectors and involvement in cat fights. No association with breed has been demonstrated (Tasker et al., 2003b; 2004a; Willi et al., 2006c; Gentilini et al., 2009; Bergmann et al., 2017).

CMt is frequently found in co-infections with other hemoplasmas, sometimes with two, three or even all species of hemoplasmas. Co-infection with C*Mhm* is the most commonly detected (Willi et al., 2006b; Peters et al., 2008b; Barker et al., 2011b; Martínez-Díaz et al., 2013). In these cases, when compared to other mycoplasmas, *CMt*'s blood concentration in any stage of the infection is the lowest and it's also the first agent to be eliminated by the organism, either spontaneously or with the use of antibiotics (Willi et al., 2006c; Peters et al., 2008b).

Up to now, C*Mhp* has only been identified in two studies, with a prevalence of 0.7% in the United States of America and 4.37% in Portugal. It was always detected in co-infection with the other species, particularly C*Mhm* (Sykes et al., 2007b; Martínez-Díaz et al., 2013). However, it was not detected in two other studies investigating the prevalence of hemoplasmosis, one in the United States of America (Sykes et al., 2008a) and the other one in Italy (Gentilini et al., 2009).

Figure 10. Prevalence of hemoplasmosis by district in Portugal (Image adapted from: http://www.ccilj.pt/portugu%C3%AAs/business-infos/sobre-portugal/).

Co-infections, both with different types of mycoplasmas (prevalence of 1-14.68%) and with retroviruses (known prevalence: FIV-19.4%/FeLV-1.5%/Both-1.5%), are associated with more severe clinical presentations (Tasker et al., 2004a; Duarte et al., 2015). The most prevalent co-infection is with C*Mhm* and *Mhf* (0.2-23.7%) (Tasker et al., 2003b; Peters et al., 2008b). In clinical presentations associated with C*Mhm* and C*Mt*, other concurrent infections are also present, while *Mhf* is typically the primary pathogen (Willi et al., 2006c; Duarte et al., 2015). Because cats have been diagnosed with all species of mycoplasmas, this suggests that there is no cross immunity between the different species and that risk factors and transmission mechanisms are similar. Infection with one single agent may also increase susceptibility to infection by the remaining (Willi et al., 2006b).

2.4 Transmission mechanism

The natural transmission mechanism of this agent is still controversial. Experimental transmission has been demonstrated by inoculating blood of infected cats using the intra-peritoneal, intravenous and oral routes (Flint, Roepke & Jensen, 1959).

There are also studies describing their transmission in cases where no arthropod vectors are present (Jensen, Lappin, Kamkar & Reagan, 2001; Tasker, 2010; Messick & Harvey, 2011).

2.4.1 Transmission via arthropod vectors

Several studies have identified that cats with outdoor access have a higher risk of infection, supporting the hypothesis that these hemoplasmas may be indirectly transmitted by hematophagous arthropods, such as fleas and ticks. In line with this chain of thought, geographic variations have been shown to have a relation, as higher infection prevalence has been detected in areas with more vegetation and with warm temperatures throughout the year, which are all favorable conditions to the activity of these vectors (Hackett et al., 2006; Willi et al., 2006c).

Ctenocephalides felis is the flea typically found in cats and several infectious agents have been isolated from them, such as *Mhf*, C*Mhm* and C*Mt* (Woods, Brewer, Hawley, Wisnewski & Lappin, 2005; Kamrani et al., 2008; Hornok et al., 2010). The simultaneous presence of these species (C*Mhm*/*Mhf* e C*Mhm*/C*Mt*) has also been detected in fleas feeding on cats with these co-infections (Lappin et al., 2006; Hornok et al., 2010). However, their role in the transmission between animals is yet to be clarified. Although the infection of a cat with *Mhf* by this route has been demonstrated, PCR positive results were transient and the cat did not develop clinical signs or changes in hemogram parameters (Woods et al., 2005). Ingestion of infected fleas and their contaminated products (eggs, larvae and feces) has also not resulted in active infection (Woods, Wisnewski & Lappin, 2006).

C*Mhm* has been identified in ticks belonging to the genus *Ixodes* and the species *Haemaphysalis flava*. These arthropods have been removed directly from cats (after feeding) and from the vegetation in the environment (Taroura et al., 2005; Willi et al., 2007b). C*Mt* has also been isolated in a tick of the species *Rhipicephalus sanguineus* (Willi et al., 2007b). However, prevalence on these studies is low, and in some cases no hemoplasmas have been identified in ticks collected from the environment. This data suggests that these arthropods have a marginal role both as an infection reservoirs and in the transmission of the infection (Willi et al., 2007b; Hornok et al., 2010). Transstadial transmission has been suggested after C*Mhm* having been identified in *Ixodes* spp. ticks that were collected from the vegetation previous to a meal (Taroura et al., 2005).

Although it was demonstrated that mosquitoes (*Aedes aegypti*) can ingest *Mhf* and *CMhm* during their meal, transmission to cats was not seen and the bacteria were not viable for long, no longer being detected by PCR 7-14 after the meal. Thus, it seems that this is not a

primary transmission mechanism for these organisms, and if they have the ability to act as a mechanic vector, this seems to be a rare event (Reagan, Clarke, Hawley, Lin & Lappin, 2017).

2.4.2 Horizontal transmission

High prevalences detected in males suggest direct transmission, which is also demonstrated in several studies where association hemoplasmosis/males and hemoplasmosis/feline infectious virus (FIV) infections have been identified (Willi et al., 2006c; Willi et al., 2007a; Sykes et al., 2008a; Bergmann et al., 2017). It's also been demonstrated that, due the their more aggressive and territorial behavior, male cats with outdoor access have higher susceptibility to infections acquired via direct contact, such as FIV, which can be transmitted via saliva and cat bites (Natoli et al., 2005; Bergmann et al., 2017). As a matter of fact, C*Mhm* and C*Mt* have been identified in the saliva and feces of infected cats up to 9 weeks after infection (AI), suggesting these may be transmission mechanisms during social interaction such as grooming, sharing food bowls or fighting (Willi et al., 2006c; 2007b; Dean, Helps, Jones & Tasker, 2008). In 2015, *Mhf* was found in the saliva, urine and feces of cats during the acute stage of the infection, albeit intermittently and in low concentrations, suggesting that, although possible, transmission via the previously described social interactions may be unlikely (Baumann et al., 2015). Museux et al. (2009) were unable to demonstrate the transmission of C*Mt* through the saliva, either using the oronasal, oral or subcutaneous routes. This could be associated with a low concentration of mycoplasmas, which was not enough for successful transmission (Willi et al., 2007b). However, they were able to demonstrate that transmission can indeed occur through bites if there is exposure to the infected cat's blood, as subcutaneous inoculation of PCR-positive blood has resulted in infection (Museux et al., 2009). As a matter of fact, abscesses secondary to fight wounds had previously been identified as a risk factor for hemoplasmosis (Grindem et al., 1990).

2.4.3 Vertical transmission

It has been suggested that the transmission of *Mhf* from queens with active infection to their offspring is possible. It is not yet clear whether this happens *in utero*, during birth or via lactogenic transmission (Fisher, Toth & Collier, 1983; Messick & Harvey, 2011). A recent example is that described in 2017, where a 10 month-old cat with no outdoor access or previous contact with dogs was diagnosed with *Mycoplasma hemocanis*. After ruling out all possibilities, the only explanation was that the parasite had been transmitted vertically (Bergmann et al., 2017).

2.4.4 Iatrogenic transmission

The transmission of hemotropic mycoplasmas via blood transfusion has already been documented (Hackett et al., 2006; Willi et al., 2006c; Duarte et al., 2015), especially in fresh blood transfusions using contaminated blood. Inherent risks of this method relate to the

viability of the agents in the blood, storage time and the ability of the receptor's immune system to control the infection (Gary, Richmond, Tasker, Hackett & Lappin, 2006; Willi et al., 2006c).

For blood collection, the commonly used solution with anticoagulants and preserving agents is citrate-phosphate-dextrose adenine (CPDA-1), which allows collected blood to be stored for up to a month (Gary et al., 2006; Graça, 2012). Gary et al. (2006) have studied the viability of *Mhf* and C*Mhm* in CPDA-1 blood during different lengths of time by inoculating hemoplasma-negative cats with this contaminated blood. They concluded that *Mhf* is viable for one hour of storage, while C*Mhm* may remain viable for a week. On the other hand, a study from Bristol University (Tasker, 2010) did not detect infection in cats inoculated with contaminated blood stored in EDTA or heparin for over one hour after collection.

In general, cats in need of a blood transfusion already have a reserved prognosis and are immunocompromised, making them more susceptible to infection, even if there is low viability and/or concentration of the infectious agents in the transfused blood (Gary et al, 2006). Therefore, it's important to perform regular molecular screening in donor cats, as has been recommended by several studies and in the consensus statement published by the American College of Veterinary Internal Medicine (Wardrop et al., 2005; Gary et al., 2006; Duarte et al., 2015). All mycoplasma species must be screened for, as their pathogenic potential isn't yet clear and may even differ between strains (Sykes, 2010a).

2.4.5 Transmission between species

Since feline, canine and rodent hemoplasmas are closely related phylogenetically, it was suggested that transmission between the different species was possible (Willi et al., 2007a).

Lumb (2001) concluded that cats may be carriers of the latent form of *Mycoplasma haemocanis* (*M. haemocanis*), as, although they don't develop infection when inoculated with this agent, they can transmit it to susceptible dogs when their contaminated blood is transfused. On the other hand, the reverse does not happen, suggesting that dogs are not susceptible to *Mhf* and are not carriers, since inoculation of their blood does not lead to infection in cats.

Results from preliminary studies involving experimental infection in cats suggest that *CMhp*, a canine parasite, can infect felines (Sykes et al., 2005). This was confirmed after *CMhp* having been found in two other studies, one in North America and another in Portugal (Sykes et al., 2007b; Martínez-Díaz et al., 2013). C*Mhp* has a closer phylogenetic proximity with C*Mhm* (94% when sequencing gene16S rRNA and 75% when sequencing gene *rnpB*) than with *M. haemocanis* (78% when sequencing gene 16S rRNA and 65% when sequencing gene *rnpB*) (Sykes et al., 2005). C*Mhm* and other genetically similar mycoplasmas have also been found in dogs (Kenny, Shaw, Beugnet & Tasker, 2004; Zhuang et al., 2009). Because of this close proximity and the fact that both have been identified in both dogs and cats, it

was proposed that this is the same microorganism infecting different species (Zhuang et al., 2009).

Due to the similarities between C*Mt* and rodent mycoplasmas, particularly *Mycoplasma coccoides* and *Mycoplasma haemomuris*, Willi et al. (2007b) looked for C*Mt* in a population of rodents captured in Zurich, Switzerland. Their results did not demonstrate that rodents are a reservoir for this agent as it was not isolated.

Three species of mycoplasmas (*Mhf*, C*Mhm* and C*Mt*) have also been found in 9 species of wild felines originating from Europe, Africa and North America. Although these populations had high prevalences, they did not have significant changes to their hemogram parameters. More studies are required to understand the potential pathogenicity of these bacteria in wild felines, however their role as potential reservoirs for these agents must be considered, as they may be asymptomatic carriers. (Willi et al., 2007c).

2.4.6 Other means of transmission

Although not yet reported, there is the possibility of iatrogenic transmission of these hemoparasites by manipulation blood of infected cats and via fomites, such as surgical material. This may happen when the contamination with blood has been marked, if contact occurs shortly after the event and, especially, if no disinfection, sterilization and hygiene procedures are properly applied (Tasker, 2010).

2.5 Pathogenicity

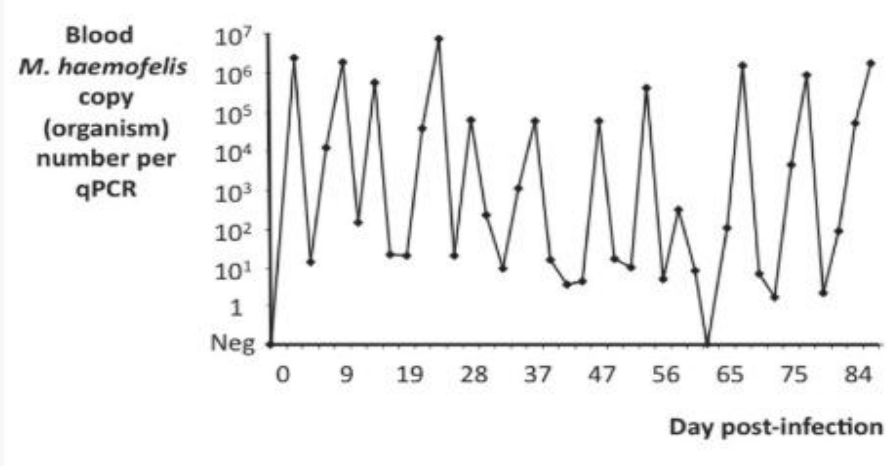
The different species of feline mycoplasmas have different levels of pathogenicity, with some isolates consistently inducing marked hemolytic anemias, while others result in few to no clinical signs. It is thought that, on top of the mycoplasma species involved, there are other factors that can affect the severity of the disease, as well as its evolution and prognosis. Some of these factors are the infecting dose, the transmission mechanism, duration of infection, age of the animal, immune system susceptibility and the presence of concurrent diseases, such as FIV or FeLV (Willi et al., 2007a; Tasker, 2010; Willi et al., 2010).

The evolution of the infection can be split in four phases: the pre-bacteremia phase, the acute phase, the recovery phase and the carrier or chronic phase (Messick & Harvey, 2011). After the experimental inoculation of *Mhf*, pre-bacteremia stage lasts for 1-34 days, depending on the used method for inoculation and agent concentration (Baumann, Novacco, Riond, Boretti, & Hofmann-Lehmann, 2013). For example, when the infection is obtained by inoculating contaminated blood ($2x10^9$ bacteria copies) intraperitoneally, this stage lasts for about 10 days (Wolf-Jäckel et al., 2010); when blood ($1{,}55x10^8$ copies) is inoculated intravenously, it lasts for about 5 days (Tasker et al., 2009a) and when the inoculation is performed subcutaneously with low bacteria concentration ($1x10^3$ copies), the agent is only detected 20-34 days after exposure. These differences likely relate to how long the bacteria

takes to reach circulation, where it then multiplies until it reaches detectable concentrations (Baumann et al., 2013).

In the acute phase, which can last for about one month, or longer if no treatment is started, bacteremia and severe anemia occur, resulting in a marked drop in hematocrit. The concentration of *Mhf* increases rapid but progressively, reaching a peak between 2 and 4 weeks AI. However, it's possible that the number of detected copies has different variations. Some cats present a cyclical progressive increase in the number of microorganisms, then a reduction in numbers, which may last for up to two hours (Figure 11). About one third of infected cats present these fluctuations. In these cases, the hematocrit levels can be inversely proportional to the levels of the microorganism, dropping progressively as the bacteria reach maximum concentration and increasing after it disappears. Except for this situation, the hematocrit remains low and may even continue to drop for one or two more days after bacteremia, particularly if there has been a marked erythrocyte destruction. Repeated episodes of bacteremia lead to the progressive lesion of red blood cells and, consequently, their time in circulation is reduced (Tasker, 2010; Messick & Harvey, 2011).

Figure 11. Changes in the number of copies of *Mhf* throughout the course of infection (Tasker, 2010).



There is no known explanation to why these changes in the concentration of *Mhf* occur, and in the case of C*Mhm* and C*Mt*, the noted changes were mild or even undetected. There are three hypothesis being discussed: 1 – The agent re-enters circulation after sequestration in organs such as the liver, spleen and lungs; 2 – Fast replication of remaining bacteria after the activation of the immune system; 3 – Evading the immune system via phase variation and/or cyclical antigen variation (Tasker et al., 2009b; Tasker, 2010; Santos et al., 2011). Although Tasker et al. (2009b) have detected a high numbers of copies of *Mhf* in the spleen and lungs, during the cycle phase, when the circulatory concentration of mycoplasmas is low, the levels detected in these organs do not justify the exponential increase in the number of copies that is observed. Rapid multiplication of the bacteria shortly after infection suggests

that this characteristic may be related. On the other hand, it was demonstrated that the majority of the genome of *Mhf* is composed by paralogous genes and tandem repeats, which is solid evidence that phase variation and/or antigenic variation could play a role in evading the host immune system, therefore being involved in cyclical episodes of the infection and its persistence (Barker et al., 2011b; Santos et al., 2011). The host organism eliminates part of the bacteria, however the ones that present different antigens, not yet identified by the immune system, persist in circulation and multiply. Despite this, the hypothesis does not explain the progressive reduction in the bacteremia cycles or its complete elimination by the organism. Three reasons for this have been proposed: 1 – The immune system produces antibodies against non-variable antigens, thus maintaining low levels of microorganisms in circulation; 2 – Antibodies produced against variable antigens may have a level of cross-reaction, partially controlling bacteremia; 3 – Prolonged bacteremia leads to the de-regulation of functional T $CD4^+$ cells, specific to the cellular response (Santos et al., 2011). As demonstrated in infections by *Anaplasma marginale*, defective production of memory T cells contributes to the persistence of bacteremia (Han et al., 2010). Groebel et al. (2009) observed that *Mycoplasma suis* can invade erythrocytes, a characteristic identified for the first time in a hemotropic mycoplasma. The bacteria increases its virulence and can resist the immune system and antibiotic treatment, persisting in the organism. This mechanism may, therefore, be one of the mechanisms responsible by concentration fluctuations of the agent, as well as the establishment of chronic infections (Rosengarten et al., 2000).

Mycoplasmas are specialized bacteria that have become obligate parasites. Genomic studies revealed that their genome is very short, not contemplating a high proportion of metabolic and catabolic mechanisms, therefore relying on host cells to obtain resulting resources such as amino acids and glucose. Because of this host dependency, the bacteria don't have a cell wall, increasing their exposure to the immune system. Since a large proportion of their genome is made of paralogous genes and tandem repeats, this suggests that there was an evolution towards developing strategies to adapt to different microenvironments and evade the host's immune response (Rosengarten et al., 2000; Barker et al., 2011b; Santos et al., 2011).

It is thought that anemia results primarily from extravascular hemolysis via erythrophagocytosis, mediated by spleen, liver, lungs and bone marrow macrophages (Tasker, 2010; Messick & Harvey, 2011). When the mononuclear phagocytic system detects erythrocytes with cellular changes or attached mycoplasmas, it can remove the bacteria or phagocyte the damaged cells, removing them from circulation and promoting their destruction. The spleen plays an important role in eliminating circulatory antigens and is also able to remodel red blood cells with lesions. This is demonstrated by the fact that infected cats, when subject to splenectomy, present bacteremias that persist for up to twice as long of

those seen in non-splenectomized infected cats (Maede, 1979; Messick & Harvey, 2011; Nelson & Couto, 2014).

The attachment of mycoplasmas to the red blood cells creates a depression in the erythrocyte cell wall, with a resulting wall erosion after the microorganism detaches. Depending on the size and depth of the erosion, the lesion may be locally repaired, result in electrolyte imbalance with subsequent osmotic fragility, or even lead to the intravascular hemolysis of the red blood cell (Jain & Keeton, 1973). This osmotic fragility has been detected in several studies (Maede & Hata, 1975; Yang et al., 2007), including in cases of C*Mt* infection (Willi et al., 2005), and it correlates with the increase in circulatory mycoplasma concentration. It was also observed that this effect persists for several days, at least up to one week after the PCR screening results turn negative. A possible explanation for this may be that part of the erythrocytes are returned to the circulation after having the mycoplasma removed by the mononuclear phagocytic system, but still have changes present on their cell membrane (Maede & Hata, 1975; Willi et al., 2005). On top of these mechanical and osmotic changes resulting from mycoplasma attachment (leading to both intravascular and extravascular hemolysis), it has also been proposed that competition for nutrients and glucose may result in early removal from circulation. The exploitation leads to reduced energy production with resulting oxidative stress and consequent reduction of time in circulation (Guimaraes et al., 2011; Santos et al., 2011). These two mechanisms may also activate programmed self-destruction of red blood cells, as has been demonstrated with *Mycoplasma suis*, contributing to intravascular hemolysis (Felder et al. 2011; Santos et al., 2011).

Coombs-positive results and the presence of auto-agglutination have been described in several studies (Maede & Hata, 1975; Tasker et al., 2009a), demonstrating the presence of antibodies linked to the erythrocytes of cats infected with *Mhf*. Tasker et al. (2009a), using a direct antiglobulin test, identified reactive antibodies both at 4ºC and at 37ºC. Cold reactive antibodies (IgM and IgG) appear between the 8th and 22nd day after infection (DAI), persisting for 2 to 4 weeks. The detection of these antibodies when anemia episodes occur suggests that the infection induces their production, however since cold agglutination does not occur at the body normal temperature, it's not yet known what their role is in the pathophysiology of the disease. IgM is very effective in the complement fixation via the classical pathway, it's possible that the deposition of complement on the erythrocytes occurs at lower temperatures in the peripheral circulation. Warm reactive antibodies (IgG) appear later, between the 22nd and the 24th DAI, persisting for up to 5 weeks. Although cold reactive antibodies are the first to be detected, they are only detected after the start of anemia. This can have two explanations, either there is no detection due to low test sensitivity at the start of infection, or the production of the antibodies is a consequence of the infection and/or of the hemolytic process itself. The attachment of mycoplasma to the erythrocytes may also lead to their

hemolysis as collateral damage, in other words, the antibodies are directed to the bacteria, however due to the proximity with the erythrocytes, they too are destroyed. *Mhf* antigen-specific antibodies have been detected up to 14 days after experimental infection. The adhesion may also expose erythrocyte proteins otherwise hidden from the immune system, or induce their alteration, causing them to be recognized and activating an immune response (Tasker et al., 2009a; Messick & Harvey, 2011). This hypothesis is also supported by documented clinical cases, where the antibodies were not detected after antibiotic and supportive treatment that did not include the use of glucocorticoids (Tasker, 2010). Therefore, even though there is a component of immune-mediated anemia, this is probably not the main cause of anemia (Tasker et al., 2009a; Tasker, 2010; Messick & Harvey, 2011). If no treatment is started, up to a third of cats infected with *Mhf* does not survive the acute phase due to the severe anemia. Cats that develop an effective immune response and have an appropriate medullary regeneration, that allows them to replenish the red blood cell pool, will enter the recovery phase. This phase takes place from the last bacteremia up to the point where the hematocrit increases to normal values or close to the normal range, and it may last at least one month. Although mycoplasmas can no longer be detected on blood smears, low concentrations of the agent may be detected by PCR, particularly if no treatment is implemented (Messick & Harvey, 2011).

Antibiotic usage has no apparent relation to the establishment of the carrier state. Carriers may be chronically infected for months, years or even their entire lifespan, or may completely eliminate the infection (Berent, Messick & Cooper, 1998; Tasker, 2010; Messick & Harvey, 2011). On top of the previously referred hypothesis that explain this, bacteria have been detected inside phagosomes, without any signs of degeneration, in spleen and lung macrophages, resulting in the proposal that some microorganisms may survive inside these cells (Maede, 1979), and that depression of the immune system (stressful events, pregnancy, concurrent infections or neoplasia) could allow the infection to reactivate (Foley, Harrus, Poland, Chomel & Pedersen, 1998; Sykes, 2010a; Messick, & Harvey, 2011; Weingart, Tasker & Kohn, 2015). However, since the study by Maede (1979), mycoplasma has only been identified in erythrocytes (Peters et al., 2011) and attempting to reactivate the infection (splenectomy, abscess formation, glucocorticoid therapy, cyclophosphamide) has not been successfully achieved, resulting only in an increase of the concentration of mycoplasma in circulation (Harvey & Gaskin, 1978)[1]. Another study documented bacteremia with a drop in hematocrit, however with no signs of anemia, by administering high doses of methylprednisolone after the patient had been asymptomatic for a period of 6 months and after finishing antibiotic treatment (Berent et al., 1998). Therefore, despite the possibility of reactivation with any of the three species of feline mycoplasmas (Weingart et al., 2015), this

[1] Quoted by: Sykes, 2010a.

seems to be a rare situation after establishment of the carrier state (Foley et al., 1998; Tasker, 2010).

Experimental infection by *Mhf* has been associated with the development of moderate to severe hemolytic anemia, and its pathogenic potential has been demonstrated in varied studies (Foley et al., 1998; Westfall, Jensen, Reagan, Radecki, & Lappin, 2001; Tasker, Helps, Day, Gruffydd-Jones, & Harbour, 2003c; Tasker et al., 2004a). However, in some studies that looked at naturally infected cats, this microorganism hasn't always resulted in anemia and it was also not possible to correlate concentrations of the microorganism with variation in hematocrit (Tasker et al., 2003b; 2004a; Willi et al., 2006c; 2007a).

There have been registered cases of healthy cats with the hematocrit within normal values that presented high blood concentrations of *Mhf* (>10^6 copies/ml of blood). On the other hand, cats with severe hemolytic anemia and a hematocrit of 12% were found to have only moderate concentrations of the microorganism (Willi et al., 2006c). These results may be explained by different susceptibilities between individuals or reflect different phases of infection. The acute phase is more closely associated with the development of marked anemias, while during the chronic phase there are no clinical signs, despite high blood concentrations of the microorganism (Willi et al., 2006c; 2007a; Tasker, 2010). It's also possible that these results are due to different cat populations being used in the different studies or even different *Mhf* strains (Tasker, 2010). Nevertheless, *Mhf* is considered the most pathogenic of feline mycoplasmas and the one with the closest association to the development of clinical disease (Tasker, 2010). Unlike most other species of mycoplasmas, including those affecting dogs and cats, this is a primary pathogen, as it does not require immunitary depression or splenectomy to cause disease (Santos et al., 2011).

CMhm infections are generally chronic and asymptomatic, with no obvious changes in hemogram parameters (Foley et al., 1998; Sykes, 2010a). Pre-bacteremia may last for 2-8 days (Foley et al., 1998; Tasker et al., 2003c; 2006; Dean et al., 2008; Tasker et al., 2009a). The number of organisms increases progressively, with a peak between 4-5 weeks, after which a plateau is detected (Figure 12) (Tasker et al., 2003c; 2006; 2009a; Sykes, 2010a). Although this mycoplasma hasn't been associated with anemia in several studies (Foley et al., 1998; Tasker et al., 2003b; 2003c; 2006; Willi et al., 2006c; Tasker, 2010; Sykes, 2010b), there have also been reported cases of acute hemolytic anemias in which the only microorganism identified was C*Mhm* (Hornok et al., 2008; Sykes, 2010a; Barker & Tasker, 2013). In other publications (Tasker et al., 2003c; 2006; 2009a), a progressive drop in the hematocrit and hemoglobin levels has been observed up to days 20-23 AI, also accompanied by an increase in the number of *agregatta* reticulocytes (Figure 13).

Figure 12. Changes in the number of copies of *Mhf*, C*Mhm* and C*Mt* throughout the infection period (Tasker, 2010).

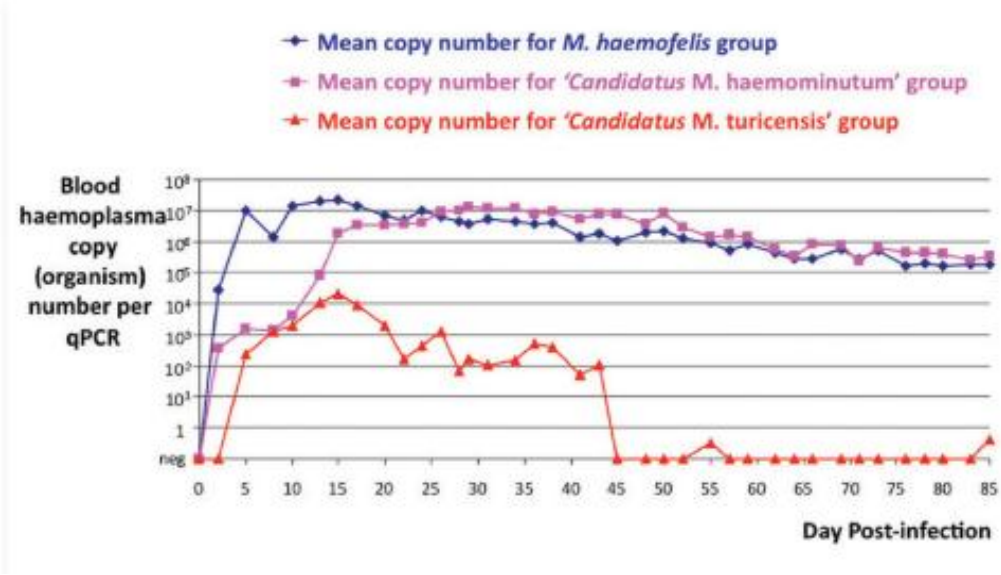
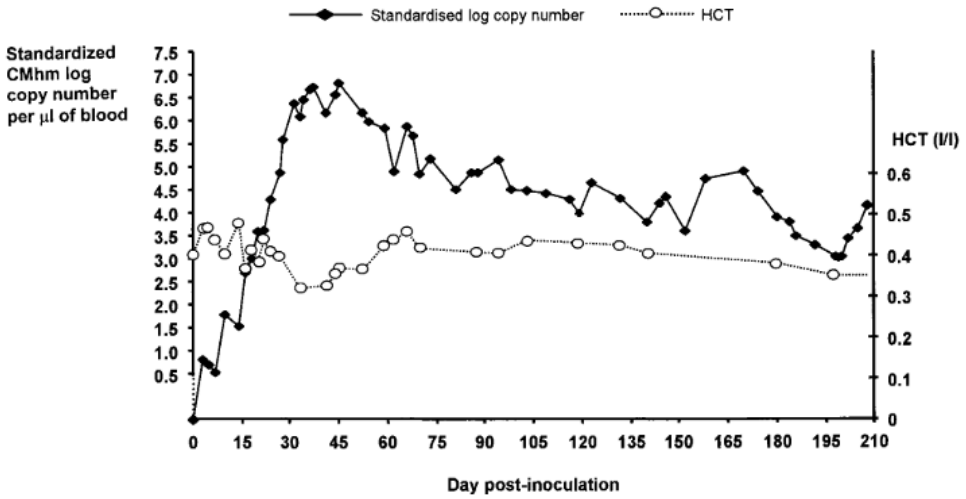


Figure 13. Changes in the number of *CMhm* copies compared with the number of days post-infection. Hematocrit levels are also represented (Tasker et al., 2003c).



These changes, which have occurred during the first month (although values remained within normal levels), suggest this mycoplasma can also cause lesions on the erythrocytes, inducing a degree of hemolysis. *CMhm* has been the species most associated with the establishment of asymptomatic carries, and it's also been demonstrated that its concentration after antibiotic treatment is similar to that of cats that have not been treated (Foley et al., 1998; Tasker et al., 2003b; 2006; Tasker, 2010; Martínez-Díaz et al., 2013), although there have been reported cases where the elimination of infection may have occurred with or without antibiotic treatment (Willi et al., 2006c). A possible explanation for these chronic infections is that the immune system is not as effective eliminating them as with the other species (Tasker et al., 2003b). Another hypothesis is that there may be a

degree of sequestrum of this species in the tissues (Wolf-Jäckel et al., 2012). The pathogenicity of this mycoplasma is therefore hard to predict, and it's possible that the changes in virulence are due to different strains, infection phases and also host immunity (Tasker et al., 2006a; Tasker, 2010; Barker & Tasker, 2013).

There is not a lot of information regarding the pathogeny of *CMt* and *CMhp* due to their lower prevalence but also the fact that they are often found in co-infections with other species of mycoplasmas or with other concurrent diseases (Sykes et al., 2008a).

Pathogenic potential of *CMt* seems to be related to co-factors and immunosuppressive states, as well as co-infections with other agents (such as retroviruses) or other mycoplasmas and concurrent diseases (e.g. neoplasia) (Willi et al., 2006b; 2006c; Peters et al., 2008b). However, there have been three reported cases where this mycoplasma was responsible for a mild to severe anemia (Willi et al., 2005). There are also reported cases of changes in hemogram parameters, despite no anemia developing. Hematocrit and hemoglobin values have been seen to drop, despite remaining within the reference range, thus demonstrating that *CMt* can also cause lesions in the erythrocytes (Tasker et al., 2009a; Tasker, 2010). This has also been demonstrated by an increase in osmotic fragility (Willi et al., 2005). After infection, pre-bacteremia can last between 5 and 11 days. The concentration of the microorganism increases and reaches the highest values between 2-3 weeks (Tasker et al., 2009a; Tasker, 2010). C*Mt* produces a low number of copies when compared to the other species, even when present in co-infections (average of 5×10^2 copies/ml) (Figure 12) (Willi et al., 2006b; 2006c; Peters et al., 2008b; Tasker et al., 2009a). It then progressively drops, regardless of antibiotic treatment, until eventually it can no longer be detected by PCR (Willi et al., 2006c; Tasker et al., 2009a; Tasker, 2010). There are records of elimination of the infection at 45 DAI (Tasker et al., 2009a; Tasker, 2010). The establishment of chronic infections is, however, possible, since the microorganisms have been detected at later points during the course of infection (Tasker et al., 2009a). This has also been demonstrated as increased concentrations were detected in body tissues, even in animals where PCR results were negative, suggesting that the low concentrations for *CMt* throughout infection, and the shorter acute phase, may be due to tissue sequestrum. This tissue sequestrum may last for long periods of time, as it's been detected for several months, even in cats where PCR results were negative (Novacco et al., 2011; Novacco et al., 2012a; Novacco, Riond, Meli, Grest & Hofmann-Lehmann, 2013). However, in other studies no reactivations have been detected, even with the administration of methylprednisolone (Willi et al., 2006c).

Regarding C*Mhp*, there is yet not enough data to allow its characterization (Tasker, 2010; Martínez-Díaz et al., 2013).

Since cats have been diagnosed with co-infections with every species of feline mycoplasma, this suggests that there is no cross protection between the different species (Westfall et al., 2001; Willi et al., 2006a; Peters et al., 2008b). It has also been observed in a study from

2015 that re-infection by *Mhf* after recovering from *CMt* results in a more severe clinical presentation (Baumann et al., 2015). The same was noted after recovering from a *CMhm* infection (Westfall et al., 2001). On the other hand, it seems that after recovering from *Mhf* infection, there is a certain level of resistance to re-infection by the same species. This was also seen in the case of *CMt* (Novacco et al., 2012a). This immune resistance seems to be mostly cellular, since plasma transfusion of cats that have recovered from the infection does not provide any protection and, on the contrary, promotes a more intense hemolysis (Hicks et al., 2015; Sugiarto et al., 2016).

There is large variation in the interaction between hemotropic hemoplasmas and the host throughout the infection, making it difficult to establish a long term prognosis (Tasker, 2010; Barker & Tasker, 2013).

2.5.1 Relation with FIV and FeLV

The role of retrovirus and their relation with mycoplasmas is not yet clearly defined, with controversial data as to their interaction and their role in the evolution of the clinical presentation (Martínez-Díaz et al., 2013; Duarte et al., 2015).

George, Rideout, Griffey and Pedersen (2002) concluded that healthy FeLV, or FIV and FeLV-positive cats, develop severe regenerative anemia after being infected with mycoplasma. It's not yet understood what process ensues because of this interaction, or if there is synergism between the two, but it has been described that causes of regenerative anemia (such as mycoplasma), stimulate erythropoiesis, resulting in macrocytosis and anisocytosis (Weiser & Kociba, 1983), and that the co-infection is associated to more severe episodes of hemolysis (Bobade, Nash & Rogerson, 1988; Harrus et al., 2002), which may explain the severity of the clinical presentation. An increase in the mitotic rate of the erythroid line, previously infected by FeLV, may be responsible for the neoplastic transformation of the precursor cells, leading to myeloproliferative diseases, such as myelodysplasia and myeloid leukemia. In these cases, *CMhm* has been identified as a potentiating agent for FeLV, as the appearance of these myeloproliferative diseases only occurs after the host recovers from the acute phase of hemoplasmosis (George et al., 2002). In several studies, FeLV is frequently detected in co-infection with *Mhf,* both of them showing a higher prevalence in younger cats (Sykes et al., 2008a; Duarte et al., 2015).

It has been observed that, comparatively with FeLV, FIV has a higher prevalence and is usually found in co-infections with *CMhm.* Both are associated with intact male cats over 5-years old with outdoor access, suggesting their transmission mechanisms may be similar (Luria et al., 2004; Sykes et al., 2008a; Duarte et al., 2015). However, there is no evidence of a synergistic association between FIV and mycoplasmas, as no increase in pathogenicity or worsening of clinical disease has been detected with either (George et al., 2002; Tasker et al., 2006a; Sykes et al., 2007b).

Despite that, several studies present contesting results. In some, the presence of retrovirus has been associated with *CMhm* (Bauer et al., 2008) or with *Mhf* (Sykes et al., 2008a), while in others there has been no direct association with a particular species, just with hemoplasmas (Sykes et al., 2007b). The association of FIV and FeLV with mycoplasmas suggests that the immunosuppressive effect of retroviruses increases susceptibility to hemoplasmosis (thus making it a secondary infection) and/or that the risk factors are similar for both (Harrus et al., 2002; Luria et al., 2004). The immunosuppression may also be responsible for the conversion of latent infection into an active one (Messick & Harvey, 2011).

2.6 Clinical signs

As already referred when discussing pathogenicity, clinical signs depend on a series of different factors, such as: mycoplasma species/strain, phase of infection, immunocompetence and presence of other concurrent diseases or infections (e.g. retrovirus) (Tasker, 2006; 2010). If the anemia develops gradually, in other words, if it's a chronic anemia, clinical signs will be mild to moderate, such as weight loss. However, with acute anemia, more severe clinical signs are present, such as mental state changes and syncope. Thus, a wide range of clinical presentations is seen, from asymptomatic cats to severe acute hemolytic anemias that can be fatal, even in immunocompetent patients (Foley et al., 1998; Tasker, 2006; 2010; Sykes, 2010a; Messick & Harvey, 2011).

Figure 14. Pale mucous membranes in an anemic cat, due to acute infection with *Mhf* (Tasker, 2010).

Clinical signs of acute infection are often non-specific and non-pathognomonic for a hemoplasma infection. They are usually signs associated with anemia, such as: pale mucous membranes (Figure 14), dehydration, lethargy, weakness, tachycardia, tachypnoea, dyspnea, inappetence/anorexia, wright loss and hemic murmur. On physical examination other findings may be present, such as hepato-splenomegaly and lymphadenopathy, possibly due to extramedullary hematopoiesis and/or hemocatheresis by the mononuclear phagocyte system. Icterus is uncommon, only occurring in cases of acute and severe hemolysis. Intermittent pyrexia, though, is common, particularly in the acute phase of the infection, potentially progressing to hypothermia in the terminal stages of the disease. Some cats may present pica, a classical manifestation of iron deficiency, and start licking cement, eating soil and litter (Westfall et al., 2001; Harrus et al., 2002; Sykes, 2010a; Messick & Harvey, 2011; Barker & Tasker, 2013).

On the other hand, chronic infections are usually asymptomatic, and the anemia, when present, is mild (Berent et al., 1998; Willi et al., 2006c; Tasker, 2010).

2.7 Laboratory changes

Hematologic changes found in hemoplasmosis cases reflect the hemolysis caused by these bacteria. Therefore, a macrocytic normochromic/hypochromic regenerative anemia can be detected (Foley et al., 1998; Tasker, 2010). The regenerative response is characterized by the presence and reticulocyte count, taking into consideration that only aggregated reticulocytes should be considered as indicators of a medullary response. In blood smears with a large number of bacteria they are harder to quantify, as both reticulocytes and mycoplasmas will be stained by new methylene blue, making this method less reliable. Other regeneration indicators are polychromasia, anisocytosis and nucleated erythrocytes, which may also be observed on the blood smear. In cats, Howell-Jolly bodies have no pathologic meaning, as they are frequently found even in healthy cats (Foley et al., 1998; Tasker, 2006; Messick & Harvey, 2011).

Non-regenerative anemias may also be found. Possible explanations for this situation are: concurrent infections with retroviruses, presence of other concurrent diseases or performing blood sampling and analysis previous to the regenerative responsive being deployed by the bone marrow (2-4 days). Another hypothesis is that the release of sequestrated red blood cells leads to an increase in hematocrit, thus no longer stimulating a regenerative response. Because of this, the hematocrit isn't always a reliable indicator of the total number of erythrocytes (Foley et al., 1998; Tasker, 2006, 2010; Messick & Harvey, 2011). Its value may drop to below 20%, with reported results averaging 10-18% (Harrus et al., 2002; Lobetti & Tasker, 2004; Tasker et al., 2009a; Messick & Harvey, 2011). Hemoglobin values are also reduced (Lobetti & Tasker, 2004; Tasker et al., 2009a).

As previously referred, it's been reported that infections with C*Mhm* and C*Mt*, even when not associated with anemia, may cause a drop in hematocrit and hemoglobin values, although they remain within the reference levels (Tasker et al, 2009a).

In the case of feline carriers, the hematocrit may suffer fluctuations, however it will not typically drop below 20%. Occasionally, mild reticulocytosis, polychromasia and an increase in mean corpuscular volume may be detected (Messick & Harvey, 2011).

Changes in the leukogram are varied, thus presenting limited diagnostic value (Foley et al., 1998; Messick & Harvey, 2011). Different leukocyte lineages may present normal counts, (Lobetti & Tasker, 2004; Tasker et al., 2009a), increased counts (Harrus et al., 2002) or decreased counts (Baumann et al., 2013; Lobetti & Tasker, 2004). Monocytosis is the most commonly detected alteration (Lobetti & Tasker, 2004; Messick & Harvey, 2011).

Regarding biochemistry parameters, an increase in urea (pre-renal azotemia) and hyperproteinemia can be detected due to dehydration. Liver enzymes may also be increased due to hypoxia secondary to the anemia and/or hepatic lipidosis secondary to anorexia. If hemolysis is severe, and particularly if it's intravascular, hyperbilirubinemia, hemoglobinemia and hemoglobinuria may all be present (Harrus et al., 2002; Tasker, 2006, 2010; Messick &

Harvey, 2011). There have been no significant changes noted for glucose values. In infections with C*Mt,* glucose values were seen to decrease, however they remained within the reference values (Tasker et al., 2009a).

As explained on the pathogeny section, Coombs-positive tests and autoagglutination have also been described (Maede & Hata, 1975; Harrus et al., 2002; Tasker et al., 2009a), and associated with infections by *Mhf* (Tasker, 2006).

2.8 Diagnosis

Carrier cats, that is, those with latent infection, are clinically healthy, as there is a balance between the multiplication and the elimination of the microorganisms (Messick & Harvey, 2011). The detection of hemoplasmas may just be a concurrent finding with no relation to present diseases (Lobetti & Tasker, 2004; Messick & Harvey, 2011). As a matter of fact, several studies (Harvey & Gaskin, 1977[2] ; Willi et al., 2006c) reported that the concentration of the bacteria does not correlate with clinical signs. The same applies when some of the diagnostic techniques have negative results, as, it has previously been mentioned, there may be fluctuations on concentration levels, leading to the microorganisms not being detected at the time of sampling. Therefore, the presence or absence of hemotropic mycoplasmas in circulation has to be taken into consideration alongside the results of the physical examination and other laboratory tests (Lobetti & Tasker, 2004).

2.8.1 Cytologic diagnosis

This refers to the cytologic examination of blood smears prepared with Romanowsky stains (e.g. Diff-Quick, Wright-Giemsa and Giemsa). The diagnosis is performed by detecting the microorganisms on the surface of erythrocytes (Tasker & Lappin, 2002; Messick & Harvey, 2011). Hemotropic mycoplasmas have a diameter of 0.3-0.8µm and may present as rounded structures (cocci), ring-like structures, comma-shaped structures or as batons. They may be found individually, as a chain or, more rarely, free in the plasma if they detach from blood cells. *Mhf* has a diameter around 0.6µm and C*Mhm* about 0.3µm (Foley et al, 1998; Tasker & Lappin, 2002; Sykes, 2010a; Messick & Harvey, 2011).

This is, however, a method with low sensitivity (0-37.5%) and variable specificity (84-98%) (Tasker et al., 2003b; 2003c; Bauer et al., 2008; Barker & Tasker, 2013).

False positive results can be obtained when there are errors with the smear drying, fixation and/or staining, originating artifacts that can be confused with the mycoplasmas. The most common ones are the appearance of stain precipitation and artifacts resulting from long periods of drying. Howell-Jolly bodies, basophilic granulation and Pappenheimer bodies (iron accumulation) are other examples of erythrocyte inclusions that can lead to confusion when

[2] Quoted by: Alleman, Pate, Harvey, Gaskin & Barbet, 1999.

identifying mycoplasmas. Using a clean filtered stain helps reducing the incidence of artifacts (Tasker & Lappin, 2002; Messick & Harvey, 2011). Sensibility improvement can also be obtained using acridine orange or using direct immunofluorescence techniques, both of which requiring an electronic microscope (Berent et al., 1998; Tasker & Lappin, 2002). With Diff-Quick stain, identifying these organisms can be difficult if their blood levels are low. Healthy cats may have up to 10% of *punctate* reticulocytes, which is why the use of new methylene blue stain or any other reticulocyte stain is not advised, since it makes it more difficult to differentiate between precipitated ribosome material and hemoplasmas (Messick, & Harvey, 2011).

Although hemotropic mycoplasmas may reach high concentrations in the blood stream, their elimination may also be swift. For example, the percentage of infected erythrocytes may drop from 90% to less than 1% in less than 3 hours (Harvey & Gaskin, 1977)[1]. This is particularly relevant for *Mhf* due to its fluctuations in concentration during the acute phase of the infection.

Therefore, even if the mycoplasmas are not identified in blood smears, the diagnosis of hemoplasmosis cannot be ruled out (Sykes, 2010a; Messick & Harvey, 2011).

In order to overcome this issue, it's recommended that several samples and smears are performed during a period of 24 hours or over several days, so as to increase the probability of finding the microorganisms in circulation (Tasker & Lappin, 2002). C*Mhm* is rarely seen in the chronic phase and C*Mt* has never been detected using optic microscopy, likely due to the low concentrations of the latter (Willi et al., 2006c; Sykes, 2010a). Although some morphologic changes have been described, (Foley et al., 1998), distinction between *Mhf* and C*Mhm* is very hard or even impossible. Another factor that may contribute to false negative results is blood storage using EDTA. This anticoagulant may cause the microorganisms to separate from the erythrocyte membrane, making their identification more challenging. Delayed preparation of the smears will aggravate the problem and may also increase the amount of precipitated content (Alleman et al., 1999; Tasker et al, 2003b; Bauer et al., 2008; Messick & Harvey, 2011).

If the smear is well prepared and the observer is experienced, the identification of these microorganisms during the acute phase of infection may be a useful tool to reach a rapid diagnosis. However, due to its limitations (Table 1), this method is becoming less frequent and the results should always be confirmed using molecular diagnostic techniques (Willi et al., 2007a; Tasker, 2010).

[1] Quoted by: Sykes, 2010a.

Table 1. Cytologic inaccuracies for detection of hemotropic mycoplasmas in cats.

Reasons	Appearance	Solution
False Positives		
Stain precipitate	Above focal plane, larger and denser-staining consistency, variable size	Use fresh-filtered stains
Drying artifacts	Irregular borders, refractile on focusing	Make thin smears, dry rapidly
Howell-Jolly bodies	Large size (1-2 µm) nuclear remnants	None
Siderotic inclusions	Focal fine, blue-staining granules	Positive with Prussian blue stain
False Negatives		
Transient bacteremia	No organisms seen despite regenerative anemia	PCR assay
Excess amount or exposure time to EDTA	No organisms seen despite regenerative anemia	New blood specimen, fresh smears, heparin or no anticoagulant

2.8.2 Molecular diagnosis

PCR amplifies specific DNA fragments, thus making them detectable. Up to this point, PCR techniques developed to identify feline hemoplasmas amplify gene 16S rRNA (Willi et al., 2007a; Tasker, 2010). As previously mentioned, using this gene may present some limitations in cases of close phylogenetic proximity, in other words, it may not be effective to detect or differentiate between different strains or species. (Fox et al., 1992; Nübel et al., 1996; Täpp et al., 2003; Barker & Tasker, 2013). It can be performed using blood smears (reduced sensitivity) (Sykes, Owens, Terry, Lindsay & Pusterla, 2008b), tissue samples (Tasker et al., 2009b), or using small amounts of blood collected in EDTA tubes, since heparin may inhibit proper technique (Willi et al., 2007a; Tasker, 2010).

There are two PCR techniques available: conventional PCR (cPCR), where products of the reaction are subject to electrophoresis and interpreted from agarose gel bands, and quantitative PCR (qPCR), based on the detection of the products of reaction from emitted fluorescence (Lobetti & Tasker, 2004; Sykes, 2010a). Most cPCR can only detect and differentiate between *Mhf* and C*Mhm* (Jensen et al., 2001), but cannot differentiate C*Mhm* from C*Mhp*, or *Mhf* from C*Mt* (Sykes et al., 2007b). In 2009, Santos et al. described a *CMt*-specific cPCR. The qPCR allows the identification and differentiation of the three species of feline hemoplasmas (*Mhf*, C*Mhm* and C*Mt*), as well as to determine their concentration by quantifying the number of copies (Tasker et al., 2003c; Willi et al., 2005; Willi et al., 2006c). In comparison with cPCR, qPCR is quicker and has less likelihood of false positives, since once the test starts it is not necessary to manipulate the samples further, as all steps occur

inside the thermal cycler, reducing the risk of contamination and processing errors (Tasker, 2006, 2010). On the other hand, it may have lower sensitivity, since the Taqman probe, despite high specificity, cannot detect variations that may occur between different species or strains (Sykes et al., 2007b; Sykes, 2010b). There are also duplex qPCR techniques available, which, on top of detecting gene 16S rRNA, also detect and amplify the feline gene 28S rRNA. This additional gene works as internal control, ensuring that there are no false negative results due to the presence of inhibitors or other flaws that may take place during processing (Peters et al. 2008b; Santos et al., 2009; Tasker et al., 2009b; Barker & Tasker, 2013). Since qPCR allows the quantification of hemoplasma DNA in circulation, it helps determining if their presence may or may not be responsible for the disease. On top of that, it also allows to monitor the evolution of infection throughout the course of treatment (Tasker, 2010; Willi et al., 2007a). If the concentration of the bacteria is too low for detection with this technique, there may false negatives. This may happen if the animal is undergoing antibiotic treatment at the time of sampling (Tasker et al., 2006b) or in case of chronic infection (Foley et al., 1998; Tasker, 2006; Tasker et al., 2009b).

It has been demonstrated in several studies that PCR is more sensitive and specific than cytologic diagnostic, having been considered the standard method (Foley et al, 1998; Jensen et al., 2001; Westfall et al., 2001; Willi et al., 2006b).

2.8.3 Immunologic Diagnosis

The inability to culture hemotropic mycoplasmas in vitro has been a major challenge in the identification and characterization of host-recognized proteins, which are the basis for these diagnostic tests (Hoelzle et al., 2007a; Peters, Helps, Gruffydd-Jones, Day & Tasker, 2010). However, the presence of an immune-mediated response has been demonstrated. By using indirect immunofluorescence, IgG antibodies have been identified up to 21 DAI, persisting for at least 6 months, even after the resolution of clinical signs (Foley et al., 1998). In 1999, using the Western blot technique, 5 antigens were identified and seroconversion is detected from 14 DAI on, persisting throughout the entire study time (60 DAI) (Alleman et al., 1999). In 2007, the first recombinant antigen (*Mycoplasma suis* HspA1) is developed and used in an ELISA (Hoelzle et al., 2007a). The monitoring of seroconversion in 13 *CMt* experimentally infected cats was performed in 2009 using a Western blot where an *Mhf* recombinant antigen was used. This protein was a truncated version of the *Mhf* heat shock protein DnaK, an analog to the HspA1 antigen of *Mycoplasma suis*. Seroconversion was identified 3-4 weeks AI (Hoelzle et al., 2007a; 2007b; 2007c; Museux et al., 2009). Genomic studies allowed sequencing and characterization of the DnaK protein gene, which was then produced as a recombinant protein and used in several ELISA tests (Barker et al., 2010; Wolf-Jäckel et al., 2010). This antigen has been used in several studies to monitor seroconversion of infected cats with either species of feline hemoplasmas (*Mhf*, C*Mhm* and C*Mt*). Antibodies are detected from 8-14 days after PCR demonstrates a positive result. They may persist for long

periods of time in circulation, sometimes even for years (1-2 years) despite negative PCR results, suggesting stimulation of the immune system during the chronic phase of infection. With the use of antibiotics, a gradual reduction is detected (Barker et al., 2010; Wolf-Jäckel et al., 2010; Novacco et al., 2011; Wolf-Jäckel et al., 2012; Novacco, Wolf-Jäckel, Riond & Hofmann-Lehmann, 2012b; Baumann et al., 2013; Novacco et al., 2018).

Further studies are yet to be carried away to test, for example, its application in naturally infected cats, whether there are cross reactions with other pathogens and characterize the immune response during the acute and chronic infection (Barker et al., 2010). Up to now, there are no known serologic techniques available (Barker et al., 2010; Peters et al., 2010; Wolf-Jäckel et al., 2010).

2.9 Treatment

Treatment should only be started in cats with clinical changes. In chronic carriers with no signs of infection, this is not recommended as no antibiotic treatment definitely eliminates the agent consistently. Treatment is also indicated when there are infections, other concurrent diseases or if the patient is undergoing immunosuppressive treatment, since recurrence of hemoplasmosis may take place and lead to clinical signs (Tasker, 2006, 2010).

2.9.1 Antibiotics

There is no antibiotic treatment that consistently eliminates hemoplasmas from circulation. This may be related to different susceptibilities of the three feline mycoplasma species, as well as other factors that may contribute to development of resistance (Tasker, 2010). An example of this was the discovery that *Mycoplasma suis* can penetrate into the erythrocytes and evade antibiotics (Groebel et al., 2009). Administration route may also play an important role. An initial intravenous administration may help maximize the antibiotic concentration in circulation, increasing its efficiency and ability to eliminate the bacteria. Although sometimes there are refractory cases to the used antibiotics, a positive clinical response is typically noticed (Tasker, 2010). There are some studies testing efficacy of protocols with sequential use of different antibiotics (e.g. doxycycline and marbofloxacin), with variable results (Novacco et al., 2012b, 2018).

2.9.1.1 Tetracyclines

Doxycycline (10mg/kg SID PO) is a commonly first line antibiotic. Treatment should take place for at least 2 weeks, however it's recommended to continue it for up to 4 weeks to try to completely eliminate the microorganism (Tasker et al., 2004b; Tasker, 2010; Novacco et al., 2018). There are, however, authors that recommend an even longer treatment of up to 6 weeks (Tasker, 2006; Barker & Tasker, 2013) or even 8 weeks (Tasker, 2010). Although studies relating to the efficacy of doxycycline refer mainly to *Mhf,* clinical cases and different observations from non-published cases suggest it's likely efficient in infections with the other

species (Tasker, 2006, 2010). Two studies using C*Mhm* had different results. Treatment with this antibiotic led to the elimination of infection in a cat that was undergoing chemotherapy for lymphoma (Lourimier & Messick, 2004)[3], but in another study, it was not effective despite 3 weeks of treatment (Sykes, Henn, Kasten, Allen & Chomel, 2007a). The same was not observed for C*Mt,* in which elimination was obtained (Willi et al., 2006c; Novacco et al., 2012b). It has been anecdotally reported that, for cats that vomit with the treatment, the dose can be split in two daily doses of 5mg/kg, improving tolerance to the drug (Tasker, 2010). Tetracyclines have, however, been associated with esophagus stenosis. Certain formulations, such as those using doxycycline hyclate, dissolve into a highly acidic solution, which may cause mucosal irritation in the esophagus that lead to esophagitis and may evolve to ulceration and stenosis. Presentations with larger sizes also take longer to reach the stomach, spending more time in the esophagus and therefore increasing the chances of this side effect. It is therefore recommended that the administration of this drug is done with food or water, which can be administered with a syringe. Another alternative is to crush the tablet and dissolve it in water, or search for a liquid presentation. Doxycycline monohydrate dissolves slower in neutral solutions, such as those found in the esophagus lumen, and is less acidic, therefore being associated with less side effects (German et al., 2005; McGrotty & Knottenbelt, 2002).

Oxytetracycline is also effective and can be used at a dose of 22mg/Kg TID PO (Tasker & Lappin, 2002; Tasker, 2010).

2.9.1.2 Fluoroquinonlones

Enrofloxacin is an effective antibiotic in the treatment of hemoplasmosis, both when it comes to resolution of clinical signs as to reduction of microorganism concentration, making it an alternative to doxycycline. Recommended dose is 5mg/kg SID PO for 2 weeks (Tasker et al., 2004b). Its use is, however, avoided in cats due to its side effects. Enrofloxacin has been associated with the development of diffuse retinal degeneration and acute blindness (Gelatt et al., 2001; Hampshire et al., 2004). Although it is noted that side effects are rare and that this is an idiosyncratic reaction, care is advised when using enrofloxacin in cats and it's not recommended for use in doses above 5mg/kg/day (Gelatt et al., 2001; Tasker, 2006, 2010).

There are two studies monitoring the effect of marbofloxacin (2mg/kg SID PO) over a period of 4 weeks. A reduction in microorganism concentration was seen, with *Mhf* demonstrating intermittently negative PCR results (Tasker et al., 2006b). In the case of C*Mhm*, its concentration hit a plateau, increasing again after treatment was stopped (Tasker et al., 2006a). In another study (Novacco et al., 2012b), marbofloxacin was used as the first line antibiotic in cats infected with C*Mt,* at a dose of 2mg/kg SID PO over 10 days. Although there was a drop in the number of microorganism copies, there was never a negative PCR

[3] Quoted by: Tasker et al., 2006a.

detected. A negative PCR was only seen when the antibiotic treatment was swapped to doxycycline. Up until now, there have been no reported side effects from the use of marbofloxacin, which was demonstrated to be effective in the resolution of clinical signs and improvement of hemogram parameters (Ishak et al., 2008; Tasker, 2010).

Pradofloxacin has also demonstrated positive results, both regarding the resolution of clinical signs and the control of the number of circulating copies of *Mhf.* It also appears to be more effective than doxycycline in eliminating the circulating microorganisms. Therefore, this antibiotic may be a good option as a second line antibiotic or in refractory cases. Studied dose was 5-10 mg/kg SID PO for 14 days (Dowers, Tasker, Radecki & Lappin, 2009).

2.9.1.3 Imidocarb

Although imidocarb dipropionate has been shown to be effective in some clinical cases, when used in controlled studies it did not result in resolution of clinical signs or improvement of hemogram parameters. However, a drop in the number of *Mhf* and *CMhm* detected by PCR was noted. This antibiotic may be considered in cases refractory to tetracyclines and/or fluroquinolone treatment. Recommended dose is 5mg/kg IM every two weeks, for a maximum of 2 to 4 injections (Lappin, Brewer & Radecki, 2002[4]; Woods, Brewer, Radecki & Lappin, 2004).

2.9.1.4 Azithromycin

In a study where azithromycin was used in infections by *Mhf* and C*Mhm*, it did not reveal to be effective to improve clinical signs or reduce the number of PCR-detected microorganisms. Its use is, therefore, not recommended (Westfall et al., 2001).

2.9.2 Corticosteroids

Using corticosteroids in the treatment of hemoplasmosis is a controversial option. Some authors recommend its use in conjunction with antibiotics, due to the fact that the resulting anemia has an immune-mediated component (VanSteenhouse, Millard & Taboada, 1993)[4]. However, up until the moment, there are no studies to prove its use is beneficial. On the contrary, their use is common in order to increase the circulating concentration of hemoplasmas and reactivate latent infections (Tasker, 2006; Willi et al., 2007a; Sykes, 2010b; Tasker, 2010; Baumann et al., 2013) and they've also been seen to worsen the infection, leading to more severe anemias (Willi et al., 2005). Concurrent infections with herpesvirus or calicivirus may also be exacerbated (Willi et al., 2007a). Their use is also questioned because anemic cats with positive Coombs tests improved just with the use of antibiotics (Tasker et al., 2009a).

Therefore, the use of corticosteroids is recommended only in cats in which the clinical signs are worsening despite appropriate antibiotic treatment, when there is a severe immune-

[4] Quoted by: Tasker, 2010.

mediated hemolytic anemia or when it's not been demonstrated that hemoplasmas are responsible for the signs of disease (Willi et al., 2007a; Tasker, 2010). In these cases, the recommended drug is prednisolone at a dosage of 2-4 mg/kg SID during antibiotic treatment, weaning it off once the antibiotic course is finished (Tasker, 2006; Willi et al., 2007a).

2.9.3 Supportive treatment

Cats presenting with acute hemoplasmosis are typically dehydrated. In this situation, fluid therapy should be used to correct dehydration. In cases of inappetence or anorexia, food ingestion should be stimulated or forced. Usually cats recover their appetite shortly after treatment is started. Since hemotropic hemoplasmas may be transmitted by arthropod vectors (e.g. fleas), it's recommended to institute treatment against external parasites in order to avoid re-infections and prevent transmission to other animals. If the anemia is severe (hematocrit below 10-12%) or if the onset of anemia is sudden, a blood transfusion should be performed using red blood cell concentrate or supplementation with oxygen-carrying solutions (such as oxyglobin) (Tasker & Lappin, 2002; Tasker, 2006, 2010; Barker & Tasker, 2013). Oxyglobin should only be used when there is no blood or derivates for a transfusion. It is a potent colloid, so it must be used carefully in cases of predisposition for hypervolemia, such as cardiac, respiratory, and renal diseases, particularly if it's used in conjunction with blood transfusions or crystalloid infusions. Recommended dose in cats varies according to different sources and is reported at 5-40ml/kg, by slow intravenous administration at a rate of 0.5-5ml/kg/h (Weingart & Kohn, 2008; Tasker, 2010).

2.9.4 Treatment monitoring

Monitoring response to treatment should be done taking into consideration reversal of clinical signs, return of hematologic tests to reference values and PCR results. After treatment is started it may take days or even weeks for the concentration of mycoplasmas to drop down to levels that are too low to be detected by PCR. One to two weeks after starting antibiotic treatment, fresh samples should be collected and analyzed. If the number of microorganism copies doesn't gradually drop, it means that the treatment is ineffective. Otherwise, treatment should continue for at least 8 weeks, finishing ideally when PCR results are negative. It should be taken into consideration that with *Mhf,* since there are fluctuations in its concentration, it shouldn't be assumed that a drop in the number of copies indicates effective treatment. As previously referred, negative PCR results don't necessarily relate to the absence of the microorganism, as the levels may simply be too low to be detected at that moment. Monitoring should take place monthly and for the first three months, and consistently negative results suggest elimination. If this is not possible, monitoring should be performed based on clinical presentation and with the goal of reducing the number of microorganism copies as much as possible. While the patient presents PCR-positive results,

infection reactivation may take place, although this is infrequent once the carrier state has been established (Tasker et al., 2006a, 2006b; Tasker, 2010).

2.10 Role in Public Health

There are several published cases of infections similar to hemoplasmosis in immunosuppressed humans. However, the diagnosis was performed based on cytologic examination of blood smears, which is associated with low sensitivity and specificity. Attempts to try to amplify the DNA of all known species of mycoplasma from these blood smears have not succeeded, indicating that cytologic diagnosis is not reliable and that those results should be carefully analyzed (Tasker et al., 2010; Tasker, 2010; Barker & Tasker, 2013). In Brazil, a co-infection by *Mhf* and *Bartonella henselae* in a human patient was diagnosed. The person lived with three cats infected with *Mhf* and had frequent injuries from feline scratches and bites. He was also immunocompromised due to an infection by acquired immune deficiency syndrome (dos Santos et al., 2008). In China, an alarming prevalence of hemoplasmosis has been noted, both in humans and in animals. It has even been reported that a group of workers from a pig farm were infected by *Mycoplasma suis* (Yang, Tai, Qiu & Yun, 2000; Hu, Yin, Shen, Kang & Chen, 2009; Yuan et al., 2009). In 2010, a clinical case was reported where an American veterinarian was diagnosed with an infection by *Mycoplasma ovis*. He had been diagnosed with multiple sclerosis and was receiving immunosuppressive medication, and he also had an infection by *Bartonella henselae*. Due to his profession, he had a history of being injured by different animal species (Sykes, Lindsay, Maggi & Breitschwerdt, 2010). In 2011, a new species of mycoplasma affecting humans was identified. The species is associated with hemolytic anemia and pyrexia and was responsive to the treatment normally used for hemoplasmosis (doxycycline) (Steer et al., 2011). These studies suggest that mycoplasmas may have zoonotic potential which is yet to be studied. Although the infection apparently manifests only in case of immunosuppression, preventative measures should be in place to prevent and limit transmission of hemoplasmosis among animals and also to the Human Being, particular for those that are involved with animals on a daily basis (e.g. veterinarians, farm workers) or with material that may be contaminated, such as blood and organic tissues (Sykes, 2010b; Tasker, 2010; Messick & Harvey, 2011; Maggi et al., 2013).

3. Experimental work

3.1 Importance

Clinical importance of hemoplasmosis is difficult to evaluate due to varied virulence (both between species and between strains), different microorganism loads and immunocompetence of the host. Their ability to establish chronic infections, also make it more complicated to assess clinical relevance, since asymptomatic animals may test positive to PCR without active infection. Negative PCR results also do not confirm the elimination of the agent just that their concentration may be too low to be detected. Carrier animals may suffer a reactivation of the infection in case of immunosuppression, and the infection may act synergistically with other pathogenic agents, worsening the clinical presentation. Hemotropic mycoplasmas cannot be cultured in vitro and this has been a challenge for their characterization and the study of their proteomic, which would help the development of a reliable serologic diagnostic method. The interpretation of an ELISA test, in parallel with the PCR, would allow a quick diagnosis and would give us the ability to know if feline hemoplasmas are present in a latent form that could be useful to assess risk of recurrence by institution of immunosuppressive treatment, allowing preventive measures, such as antibiotic treatment. Wolf-Jäckel et al. (2010) published the first study relating to the identification, characterization and expression of the *Mhf* gene coding the rDnaK a heat shock protein, analogous to antigen HspA1 of *Mycoplasma suis*, found in the cell membrane of the bacteria. The rDnaK peptide was successfully used in Western blot and ELISA techniques to detect antibodies in samples collected from experimentally infected cats.

3.2 Objective

The objective of this study was the evaluation of this new recombinant protein in an ELISA (rELISA) technique for the detection of *Mhf*-specific antibodies in naturally infected cats. Simultaneously, the specificity and sensitivity of this technique was compared with the molecular diagnostic by qPCR and cytologic diagnostic, that were also performed.

3.3 Materials and methods

3.3.1 Selected population and sample collection

Within the scope of the TNR program performed by the Municipal Council of Lisbon to control the population of feline colonies, a sample of 1 ml of total blood, collected from the jugular vein, into an EDTA tube, was obtained from a total of 104 colony cats subject to neutering. All of the animals come from Lisbon City Council, with 46 being females and 58 being males. Because of the specific conditions of this program, it was not possible to obtain information regarding the health condition of these animals or perform a follow up.

This project was approved by the FMV/University of Lisbon ethics committee (CEBEA).

3.3.2 Sample processing

Each blood sample collected (1 ml) was split into three aliquots:

1 - 500µl were centrifuged to obtain plasma, which was then sent to the laboratory of the Vetsuisse Faculty, for the detection of specific antibodies as previously described (Wolf-Jäckel et al., 2010);

2 - 200µl were used to perform total DNA extraction, in order to perform molecular diagnosis by qPCR, in the Virology Laboratory (CIISA, FMV/Ulisboa);

3 – The remaining 300µl were centrifuged at 1500xg for 10 min, and the plasma and the cellular pellet were both stored at - 80ºC.

3.3.3 Extraction and quantification of nucleic acids

Total DNA extraction from the blood samples was performed using the commercial kit DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer instructions.

1º 20µl of proteinase K and 100µl of PBS (for volume adjustment) were added to 200µl of total blood. Mixing was made by vortexing;

Figure 15. Reagents used in DNA extraction.

2º 200µl of denaturing buffer (AL) were added, the sample was mixed and incubated at 56ºC for 10 minutes;

3º After incubation, 200µl of absolute ethanol were added and mixed to promote precipitation of DNA in the solution, which was then transferred to a silica column, to which DNA was adsorbed after centrifugation at 16000xg for 1 minute;

4º Two washes were then performed with 500µl of the wash buffers AW1 and AW2 as recommended by the kit. The samples were re-centrifuged for 3 minutes in the same conditions described on the previous step;

5º Finally, DNA was eluded from the column by adding 200 µl of the elution buffer AE and centrifuged into a new sterile microtube;

6º After these steps, DNA was quantified in the spectrophotometer Nanodrop 2000c (Thermo Scientific) and stored at -20ºC until further use.

3.3.4 Detection of nucleic acids

The technique used to detect the presence of feline hemotropic mycoplasmas was real-time PCR (qPCR), using a TaqMan® probe (Applied Biosystems), which includes a pair of primers outlining the target area for amplification and a specific probe marked with a fluorophore (*reporter*) on the 5’ end and a *quencher*, or suppressor, on the 3’ end. While the probe does not link to the corresponding DNA region, outline by the forward and reverse primers, the quencher and the reporter remain close, which leads to the inhibition of the reporter’s fluorescence by the quencher. After the probe and primers connect to the target

sequence, the process of polymerization of the new chain starts and the 5'-3' exonuclease activity of the Taq polymerase hydrolyses the probe, leading to the separation of the reporter and the quencher with subsequent fluorescence emission. The intensity of the emitted fluorescence at the end of each cycle is proportional to the amount of amplified product, which is presented in a graphic that allows real-time monitoring of the kinetics of the amplification reaction. If the target sequence is not present in the sample, the probe does not link and, consequently, there is no fluorescence (Figure 16) (VanGuilder, Vrana & Freeman, 2008).

Taqman probes have been used in the detection of *Mhf* and C*Mhm* (Table 2). In the case of *Mhf*, the *primers* and the probe were calculated using the program *Primer designing tool* from NCBI – (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), based on the nucleotide sequence of gene 16S RNA (Duarte et al, 2015). Primers and probe used for the detection of C*Mhm* were those referred by Tasker et al., (2003c). For the detection of C*Mt* the system referred by Willi et al., (2005) was used, in which a probe with a conjugated *Minor Groove Binder* at the 3' end had been designed. This molecule allows a superior Tm for smaller probes, ensuring greater sensibility and specificity (Table 2) (VanGuilder et al., 2008)

Figure 16. Amplification curves of qPCR for each mycoplasma species: a – *Mhf*; b – C*Mhm*; c – C*Mt*

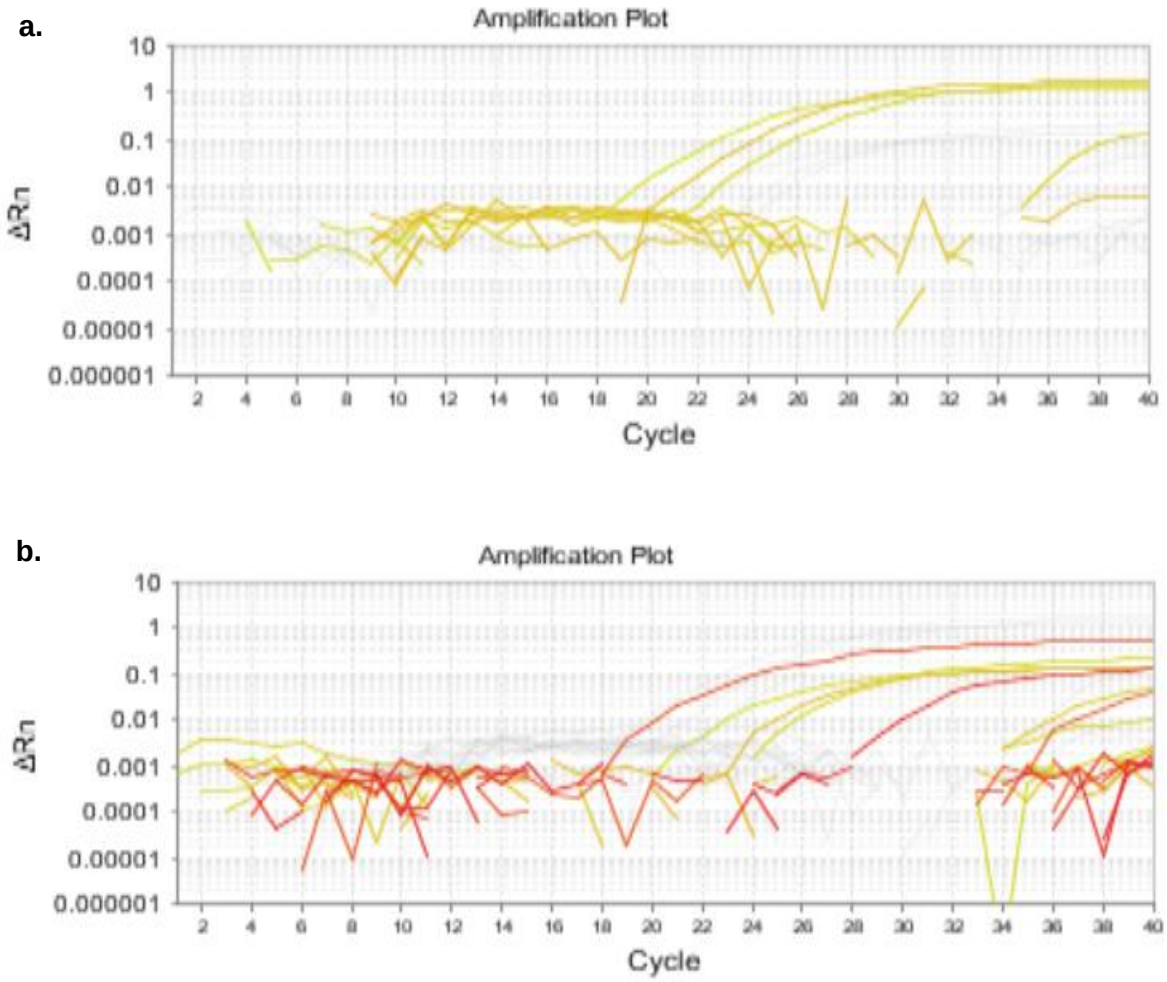


Figure 16 (cont.). Amplification curves of qPCR for each mycoplasma species: a – *Mhf*; b – C*Mhm*; c - C*Mt*

c.

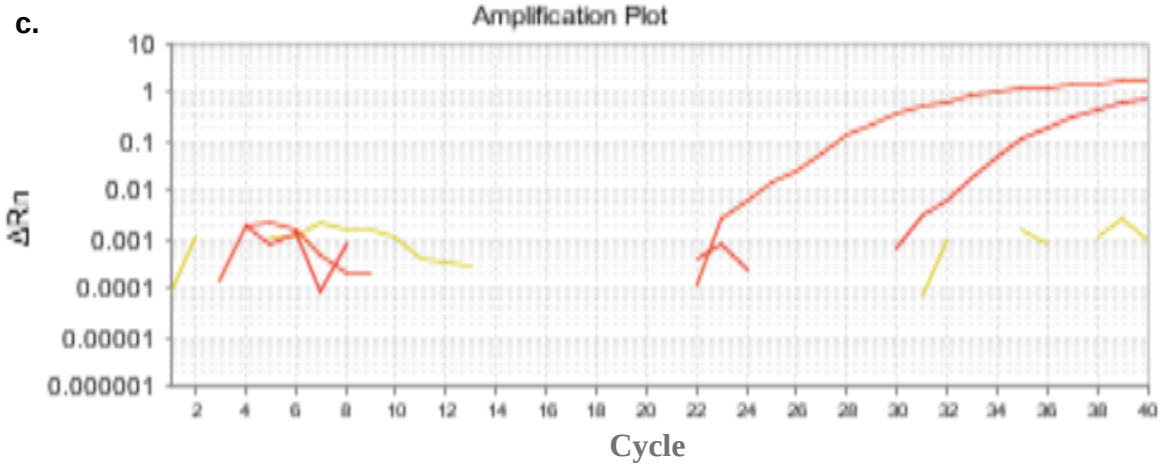


Table 2. Nucleotide sequence of the used primers and probes.

Species	Sequence (5'- 3')	5' Reporter/ 3' Quencher
Mhf [1]	Forward CGG CCA AGG TTA GTG GCA AAC GG	FAM/TAMRA
	Reverse TCC CTCA GCG CCC GAA GGC T	
	Probe FAM ACA TGC CCC TCT GTG GGG GAT AGC CGC TTG	
C*Mhm* [2]	Forward ACG AAA GTC TGA TGG AGCA ATA	JOE/TAMRA
	Reverse ACG CCC AAT AAA TCC GRA TAA T	
	Probe JOE AGC TTG ATA GGA AAT GAT TAA GCC TTG AA	
C*Mt* [3]	Forward GAA GGC CAG ACA GGT CGT AAA G	FAM/MGB
	Reverse CTG GCA CAT AGT TWG CTG TCA CTT A	
	Probe FAM AAA TTT GAT GGT ACC CTC TGA	

[1] Duarte et al, 2015

[2] Tasker et al., 2003c;

[3] Willi et al., 2005.

The protocol used for the detection of the three species of hemoplasmas was similar. For a reaction volume of 20 µl, 2x *TaqMan® Gene Expression Master Mix* was used, which includes: the reaction buffer, the DNA polymerase and dNTPs, 0,9 µM of *forward* and *reverse primers*, 0,25 µM of probe and 10-50ng of DNA. Reactions were amplified in the thermocycler *One Step Plus* (*Applied Biosystems*) using a standard amplification protocol (50ºC/2min; 95ºC/15min; 50 cycles at 90ºC/15sec and 60ºC/1min).

As positive control for the reaction with *Mhf,* a recombinant plasmid available from the Virology Laboratory of CIISA, FMV-ULisboa was used. Positive samples of *CMhm* and *CMt,* previously confirmed by sequencing, were used as positive control. For negative control, a combination of PCR reagents and MilliQ® water, without DNA sample was used.

3.3.5 Preparation and observation of blood smears

Immediately after blood collection, and before storage in EDTA tubes, blood smears were performed. They were air dried, then fixed in methanol for 1 minute. The chosen stain was Giemsa, which, after filtration, was also applied for 1 minute. The samples were then washed with tap water and allowed to air dry. Observation was performed on a magnitude of 1000x using immersion oil in the microscope Olympus BX50 (Figure 17).

Figure 17. Olympus BX50 microscope used for the observation of blood smears.

Criteria to determine if smears were or not positive for mycoplasmas were the following (Figure 18 and 19):

1 – Size smaller than 1µm;

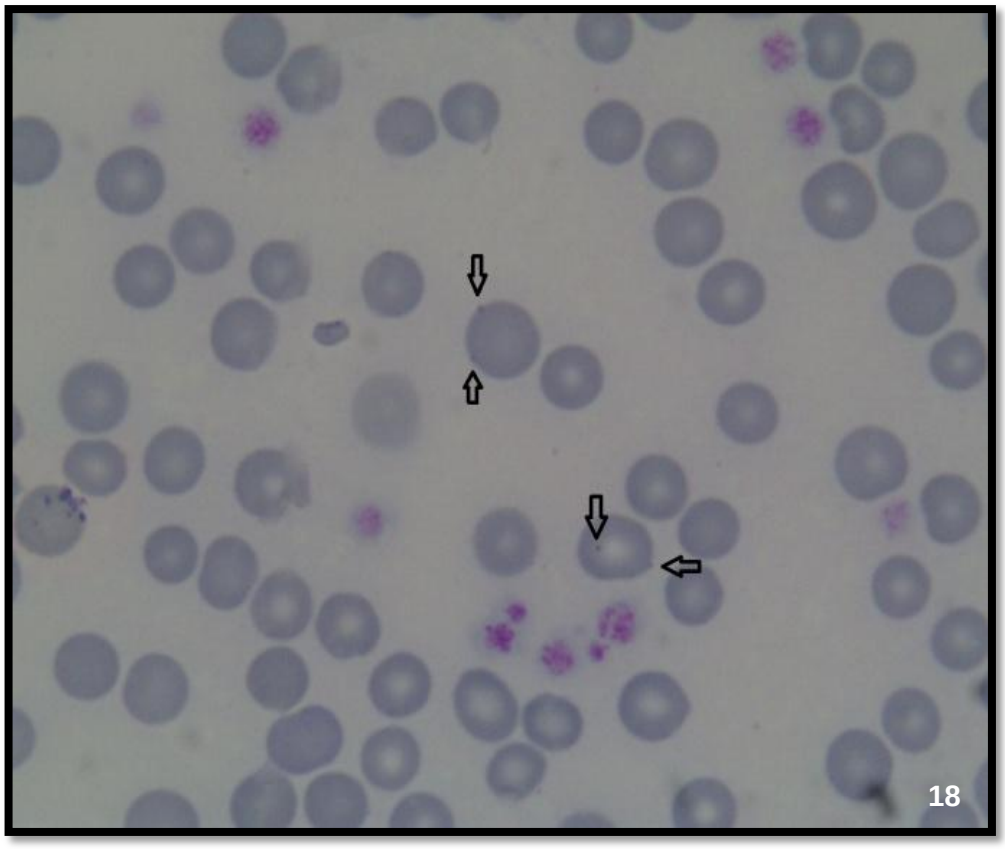
2 – Red coloration;

3 – Preference for a marginal position, that is, attached to the membrane, although they may also be seen in other locations, such as the center of the erythrocyte or detached from the cells;

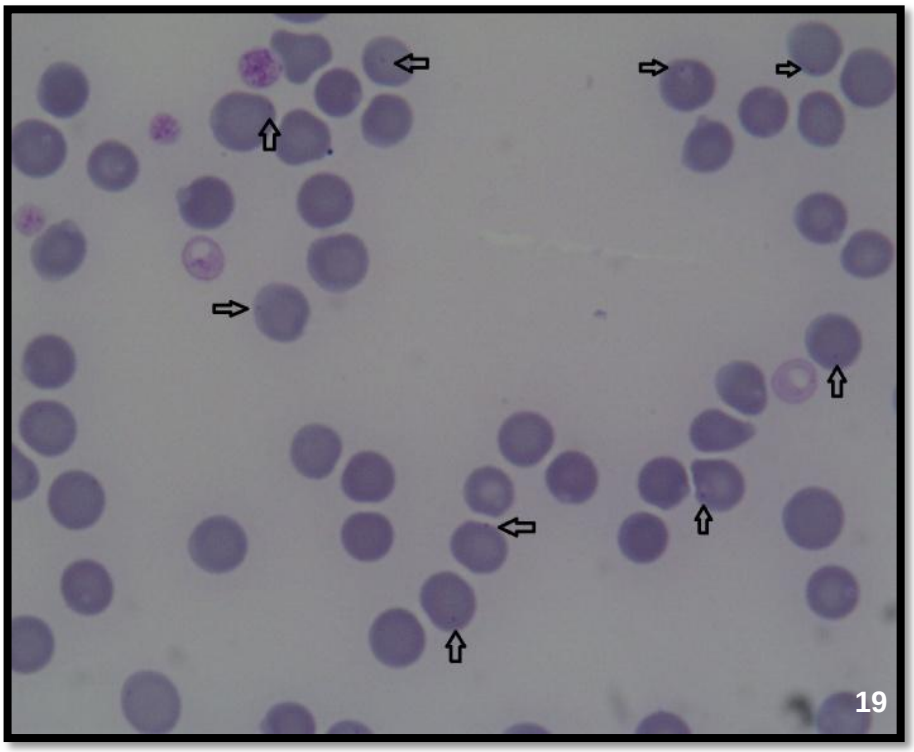
4 – It cannot present refringence and must maintain size and coloration when focus is changed;

5 – The identified shapes must be identified several times throughout the smear.

Figures 18 and 19. Examples of two smears that were considered positive. Arrows point to some of the mycoplasmas.



Figures 18 and 19 (cont.). Examples of two smears that were considered positive. Arrows point to some of the mycoplasmas.



3.3.6 Antibody detection against *M. haemofelis* DnaK

Antibodies against the recombinant DnaK protein of *Mhf* were measured in serum samples from cats using a previously described ELISA (Wolf- Jäckel et al., 2010). Briefly, sera were diluted 1:200, and 50 ng of recombinant protein per well was used. Optical density (OD) was measured at a wavelength of 415 nm using a spectrophotometer (Spectramax Plus 348; Molecular Devices, Sunnyvale, CA, USA). Serum from a non-hemoplasma exposed SPF cat and serum from an acutely *Mhf* infected SPF-derived cat (cat KCU1 from Baumann et al., 2013) served as negative and positive control, respectively.

Anti-DnaK antibodies were standardized and given as percentage of the positive control, calculated as follows: (OD value [sample]-OD value [negative control])/(OD value [positive control]-OD value [negative control]). Samples with less than 10% of the positive control were considered negatives, between 10-20% were considered borderline and above 20% were considered positives (Appendix B).

3.3.7 Data analysis

The prevalence of infection in the studied population was calculated, as well as the sensitivity and specificity of each diagnostic method used (molecular diagnostic, cytological diagnostic, serologic diagnostic).

In order to assess agreement between the different methods, the Cohen's Kappa (k) was calculated. Interpretation of the K values was performed based on the Landis and Koch method (Houe, Ersboll & Toft, 2004) (Table 3).

Both prevalence, specificity and sensitivity were determined on the website http://epitools.ausvet.com.au, as well as Cohen's Kappa (k).

Table 3. K values interpretation was performed based on the Landis and Koch method (Houe et al. 2004).

K Values	Agreement
K ≤ 0.2	Slight
0.2 < K ≤ 0.4	Fair
0.4 < K ≤ 0.6	Moderate
0.6 < K ≤ 0.8	Substantial
0.8 < K	Almost perfect

3.4 Results

According to the results of the qPCR the prevalence of hemoplasmosis in the studied population was 22.1% (23/104). Infection by *Mhf* was found in 6.7% of the animals (7/104), of which 1.9% (2/104) were single infections by this microorganism and 4.8% (5/104) were co-infections. C*Mhm* was found in 18.3% of the sample (19/104), with 11.5% (12/104) corresponding to single infections and the remaining 6.7% (7/104) to co-infections. Regarding *CMt*, it was found in 4.8% of the studied cats (5/104), of which 1.9% (2/104) were single infections and 2.9% (3/104) were co-infections (Table 4). The proportion of infected males was 29.3% (0.19-0.42 CI 95%) (17/58) and females was 13% (0.06-0.26 CI 95%) (6/46).

In the cytologic examination, mycoplasmas were observed on the blood smears of 49.1% (N=51) of the samples and 50.9% (N=53) were considered negative.

As for the rELISA results, 15.4% (N=16) of samples were seropositive, 9.6% (N=10) were considered borderline and 75% (N=78) were negative.

Of 22.1% (N=23) of samples where the microorganism was identified by qPCR, mycoplasmas were also identified on the blood smears of 15.4% (N=16), 4.8% (N=5) tested as seropositive and 2.9% (N=3) had bordeline results. Of the same 22.1% qPCR positive samples, 6.7% (N=7) were negative in cytologic examination and 14.4% (N=15) were seronegative (Table 5 and 6).

Table 4. Number of infections of each species of mycoplasmas, as well as their prevalence and confidence interval (CI).

Species	Number of positives	Prevalence (95%)	CI 95%
Mycoplasmas	23	22.1	0.15-0.31
Mhf (SI)	2	1.9	0.01-0.07
C*Mhm* (SI)	12	11.5	0.07-0.19
C*Mt* (SI)	2	1.9	0.01-0.07
Mhf (SI+CoI)	7	6.7	0.03-0.13
C*Mhm* (SI+CoI)	19	18.3	0.12-0.27
C*Mt* (SI+CoI)	5	4.8	0.02-0.11
Mhf (CoI)	5	4.8	0.02-0.11
C*Mhm* (CoI)	7	6.7	0.03-0.13
C*Mt* (CoI)	3	2.9	0.01-0.09
C*Mhm*/*Mhf*	4	3.9	0.02-0.09
C*Mhm*/C*Mt*	2	1.9	0.01-0.07
C*Mhm*/*Mhf*/C*Mt*	1	1	0.00-0.05

Legend: Co-infection (CoI); Single Infection (SI)

Of 77.9% (N=81) of samples that were negative by qPCR, only 44.2% (N=46) were also negative on cytologic examination, 60.6% (N=63) were considered seronegative and 6.7% (N=7) were borderline. Of the same 77.9% qPCR negative samples, mycoplasmas were identified on 33.7% (N=35) of blood smears and 10.6% (N=11) of samples were considered seropositive (Table 5 and 6).

Table 5. Relation between the results of qPCR and rELISA.

rELISA	**qPCR**		**Total**
	Negative	Positive	
Negative	60.6%	14.4%	75%
Positive	10.6%	4.8%	15.4%
Borderline	6.7%	2.9%	9.6%
Total	77.9%	22.1%	100%

Table 6. Relation between the results of qPCR and cytology.

Cytology	**qPCR**		**Total**
	Negative	Positive	
Negative	44.2%	6.7%	50.9%
Positive	33.7%	15.4%	49.1%
Total	77.9%	22.1%	100%

The sensibility of cytology was 69.6% (0.47-0.87 CI 95%) and specificity was 56.8% (0.45-0.68 CI 95%). rELISA had a sensitivity of 25% (0.09-0.49 CI 95%) and a specificity of 85.1% (0.75-0.92 CI 95%).

Cohen's Kappa (k) was calculated to assess agreement between qPCR-Cytology (k=0.1836) and PCR-rELISA (k=0.1093).

3.5 Discussion

The prevalence of hemoplasmosis in the studied population was 22.1%, of which 6.7% correspond to infection by *Mhf,* 18.3% by *CMhm* and 4.8% to *CMt.* These values were similar to those found by Duarte et al. (2015) that took place in the Centre and South of Portugal, with a global prevalence of 27.1%, with 14.4% corresponding to the detected presence of *Mhf,* 17.8% to *CMhm* and 5.9% to *CMt.* Detected co-infections corresponded to 8.1%. The same was observed when the prevalence was compared to that found in the council of Lisbon, which was 29.1% (Duarte et al., 2015). On the other hand, there was a marked discrepancy with another study that took place in the North and Center of Portugal (Martínez-Díaz et al., 2013). In this publication, molecular techniques were also used (qPCR) and the prevalence of infection by feline mycoplasmas was 43.43%, with a recorded prevalence of 12.81% for *Mhf,* 41.56% for *CMhm* and 1.25% for *CMt.* Co-infections were also detected with a prevalence of 14.68%. These differences can be associated with factors such as the sample size, geographic distribution, animal lifestyle and clinical status. The results on the present study did not differ significantly from those reported for Europe (Hemoplasmosis– 10-43.4%; C*Mhm* – 5.3-41.6%; *Mhf* – 1.4-12.8%; C*Mt* – 0.3-1.7%) which vary according to the reviewed bibliography (Willi et al., 2006a; 2006c; Peters et al., 2008b; Gentilini et al., 2009; Maher et al., 2010; Roura et al., 2010; Martínez-Díaz et al., 2013; Duarte et al., 2015; Rosenqvist et al., 2016; Sarvani et al., 2018). The worldwide prevalence of hemoplasmosis is 4-49.4%, and the proportion of *Mhf*, C*Mhm* and C*Mt* is 0.5-12.81%, 3.3-41.56% e 0.3-6.7% respectively (Tasker et al., 2004a; Willi et al., 2006a; 2006c; Yu et al., 2007; Ishak et al., 2007; Sykes et al., 2008a; Peters et al., 2008b; Greenwood & Prescott, 2008; Gentilini et al., 2009; Roura et al., 2010; Maher et al., 2010; Tanahara et al., 2010; Stojanovic & Foley, 2011; Bennett et al., 2011; Braga et al., 2012; Kamrani, Parreira,; Lobetti & Lappin, 2012; Jenkins et al., 2013; Martínez-Díaz et al., 2013; Vergara et al., 2016; Rosenqvist et al., 2016; Sarvani et al., 2018). The results of this study demonstrate once again that *CMhm* is the most prevalent species, followed by *Mhf* and C*Mt*. As in a previously published work (Duarte et al., 2015), the prevalence of *CMt* in Portugal was higher than that reported for other countries. Interestingly, this was not observed in the study that took place in the North and Center of Portugal (Martínez-Díaz et al., 2013).

As for gender, a higher prevalence in male cats was observed (29.3%) when compared to female cats (13%). This is in accordance with the proposition that males have a higher risk of

infection by these organisms due to their free roaming lifestyle and their aggressive and territorial behavior; when compared to females (Natoli et al., 2005; Duarte et al., 2015; Bergmann et al., 2017).

Taking into consideration the population investigated in this study, a higher prevalence of hemoplasmosis was expected. Free roaming cats and feral cats have been associated with a higher risk of infection due to their lifestyle. They are exposed to various risk factors, such as outdoor access, exposure to arthropods and fights between rival cats (Hackett et al., 2006; Willi et al., 2006a; Roura et al., 2010; Duarte et al., 2015; Bergmann et al., 2017).

As previously referred, caution must be taken when comparing prevalence studies due to geographic differences, different diagnostic methods and statistical analysis, characteristics and size of the sampled population and inclusion criteria (Bauer, Balzer, Thüre & Moritz, 2008; Gentilini et al., 2009; Messick & Harvey, 2011; Martínez-Díaz et al., 2013).

As expected, cytologic diagnostic had low sensitivity (69.6%) and specificity (56.8%) when compared to qPCR. Even though the stain was filtered to reduce the incidence of artifacts, there is always the risk of false positive results due to the incorrect identification of erythrocyte inclusions such as Howell-Jolly bodies, basophilic strippling, Pappenheimer bodies as hemotropic mycoplasmas. As blood smears were air-dried, there is also the possibility of stain precipitation or other artifacts resulting from prolonged drying times. To reduce the incidence of false negatives, blood smears were performed immediately after blood collection and before the blood was stored in EDTA tubes, as this anticoagulant can cause the microorganisms to detach from the erythrocyte membrane, making identification more challenging. However, if sampling takes place when the concentration of the microorganism in circulation is low, such as in chronic infections, false negatives can be seen. Another factor to consider is the inexperience of the observer, which is particularly relevant with these microorganism due to the associated challenges with its identification (Alleman et al., 1999; Tasker et al, 2003b; Bauer et al., 2008; Messick & Harvey, 2011; Tasker & Lappin, 2002). When compared with qPCR, this diagnostic method has a slight agreement (k=0,1836).

There isn't a gold standard test to detect *Mhf*-specific antibodies. Therefore, the presence of the agent was determined by the results of qPCR. rELISA presented a low sensitivity of 25% and a specificity of 85%. The low sensitivity may be related to phase variation and/or antigenic variation. As the genome of these bacteria includes paralogous genes and tandem repeats, they have a high capacity to modulate genetic expression, which allows them to adapt to different environments. The components with greater variability are surface components, particularly cell membrane lipoproteins. These are the proteins recognized by the immune system and induce the immune response. It has been suggested that this is the mechanism that allows mycoplasmas to evade the immune system and establish chronic infections. Therefore, the choice of antigen for a serologic diagnostic technique is particularly

important; if the chosen protein is a variable protein, there is the risk of obtaining false negatives (Alleman et al., 1999; Rosengarten et al., 2000; Santos et al., 2011; Barker et al., 2011). It is known that the DnaK protein which was used in this rELISA is a surface protein, which may be one of the possible explanations for the low number of seropositive samples (Hoelzle et al., 2007a; Barker et al., 2011).

Since it's not possible to culture hemotropic mycoplasmas in vitro, using recombinant antigens is a viable alternative. This method allows the stable production of the recombinant protein, avoiding its collection from experimentally infected animals. Recombinant antigens may however, present other problems, which can in turn interfere with the results. They may have low sensitivity, as only few different antigens from the agent are used; they can present cross-reactions due to the presence of by-products of the host bacteria *Escherichia coli* and low stability of the expressed recombinant protein. Any of these factors could be responsible for the low sensitivity and specificity obtained on this study when using rELISA (Hoelzle et al., 2007c).

Regina Hofmann-Lehmann, one of the co-authors for the article in question (Wolf-Jäckel et al., 2010), referred that due to the limited experience with field samples, the interpretation and categorization of the rELISA results may not be adequate and require future adjustment (personal communication, April 10, 2018). If this is confirmed, the results obtained on this study may need a readjustment, due to the impact on the technique's performance and agreement with qPCR assay.

PCR results that are positive but with corresponding rELISA negative results may be explained by recent infections, where seroconversion hasn't yet taken place. They may also be false negatives derived from antigenic variation and/or phase variation, that is, different strains and/or species with different antigen epitopes which are not detected. It's also possible that this single protein is not enough to detect all the cases or that it's not stable enough for use. Errors or problems with transportation of the samples to Zurich, such as fluctuations in temperature, storage conditions or long transportation period, may also have caused deterioration, which may also account for problems with the detection.

Of the 81 results that were negative by qPCR, rELISA detected seropositivity in 11 samples and 7 borderline samples. These results (qPCR negative, but rELISA positive) can be associated with cross reactions with other pathogens or byproducts of the host bacteria *Escherichia coli* related to the recombinant protein purification. Another explanation is that these may be chronic infections. The mycoplasma load is below the sensitivity of the molecular assay, despite the previous stimulation of the immune system that resulted in the production of antibodies. Similarly, the detected antibodies can result from a previous infection in which the bacteria has already been eliminated from the organism. When the rELISA was compared to the qPCR performance there was only a slight agreement between teem (k=0,1093).

Despite of these results, this antigen (DnaK) has been successfully used in several studies with experimentally infected cats. This rELISA has been used to monitor infections with the three species of mycoplasma (Wolf-Jäckel et al., 2010; Novacco et al., 2012a; Wolf-Jäckel et al., 2012; Baumann et al., 2015; Sugiarto et al., 2016), leading to the conclusion that there is a progressive drop in the antibody levels with the use of antibiotics (Novacco et al., 2012b; Baumann, Novacco, Riond, Boretti & Hofmann-Lehmann, 2013; Novacco et al., 2018). The immunosuppressive effect of corticosteroids has also been confirmed, as a temporary reduction of the antibody levels was detected after the administration of methylprednisolone (Novacco et al., 2011). In several of these studies, a correlation between the concentration of the agent and the antibody levels has been detected (Wolf-Jäckel et al., 2010; Novacco et al., 2012b; Wolf-Jäckel et al., 2012; Baumann et al., 2015). The detection of seroconversion 7 to 14 days after positive PCR results has also been reliably reproducible (Wolf-Jäckel et al., 2010; Novacco et al., 2012b; Baumann et al., 2013; 2015).

The population used in this study consisted of naturally infected cats. Due to the characteristics of the TNR program, under which the samples were obtained, it was not possible to obtain any information regarding the clinical status of these animals or whether they had other concurrent infections or diseases. There is a high risk for cross reactions or the presence of strains and/or species with different antigens. There is no possibility to assess whether the detected antibodies relate to an active infection, a chronic infection or a past infection, with the elimination of the microrganism. Without knowing the clinical status of the animals or without follow-up it's not possible to evaluate the phase of the infection based on qPCR results, since cats with high microorganisms load have been recorded as not having any clinical signs (Willi et al., 2006c).

3.6 Conclusion

Further studies are needed in order to understand and characterize the immune response towards hemotropic mycoplasmas. The non-variable antigens, preserved between species and strains, must be determined. Due to the dynamic that these bacteria present in their surface proteins, it should be taken into consideration that the use of more than one antigen in the serologic diagnosis may be of use, improving the assay sensitivity even in different phases of the infection. The possibility of cross reactions has to be assessed, as well as changes in pathogenicity kinetics and the immune response when there are other infectious agents present (e.g.: *Bartonella henselae*, *Chlamydophila felis*, FIV or FeLV). All these factors are important, particularly when the objective is to develop a diagnostic method to be used in field conditions and clinical practice, where the patients are naturally infected and the clinician is unaware of whether they also have other infectious agents in circulation. The development of an ELISA test would be helpful for the diagnosis of hemoplasma infections, but also to determine their clinical relevance and to help distinguishing acute and latent

infections by using it concurrently with the clinical signs and PCR results. It could also be useful to monitor antibiotic treatment efficacy and to search for antibodies in circulation before starting an immunosuppressive treatment, aiding with the implementation of preventative measures, such as the use of antibiotics, to avoid infection reactivation.

Hemoplasmosis is an infection that is frequently underestimated in clinical practice. Although it does not always result in anemia, it may act synergistically with other agents or co-factors, leading to the worsening of the patient's clinical status. Since the transmission mechanisms are not fully clarified, this partly limits the implementation of preventive measures, which poses a risk of infection both to other animals but also for humans. It is also necessary to rule out the presence of these microorganisms in cats admitted for blood donations in order to prevent their transmission. As there is yet no treatment that consistently eliminates hemoplasmas, further studies should take place to develop more effective protocols, thus reducing the prevalence of chronic infections. More studies are also necessary to characterize the genome of the different species of hemoplasmas, both to clarify biochemical processes, allowing the development of an appropriate culture medium that allows their growth in a laboratory setting, and also to expand the knowledge in proteomic and immunoproteomic, clarifying the pathogenicity factors and mechanisms of evasion to the immune system. This information would help in the development of serologic diagnostic techniques, but also the creation of an effective vaccine that would prevent the infection.

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5. Appendices

Appendix A – Poster presented at CIISA congress 2018

Feline hemoplasmas: evaluation of specific antibodies and the molecular and cytological diagnostic

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Introduction

Mycoplasma haemofelis (*Mhf*), Candidatus *Mycoplasma haemominutum* (*CMhm*) and Candidatus *Mycoplasma turicensis* (*CMt*) are three feline hemoplasmas, responsible for severe hemolytic anemia in felids. The laboratory diagnosis of infection can be performed by cytological examination of blood smears or by quantitative PCR assay (qPCR), which is considered the gold standard method, due to its sensitivity and specificity. For a better understanding of the infection pattern and differentiation of acute and chronic infections, an ELISA was developed (1) using a recombinant antigen (rDnaK; *Mhf*).

Objectives

Evaluate the usefulness of this recombinant ELISA (rELISA), for the detection of specific hemoplasmas antibodies in naturally hemoplasma-infected cats.

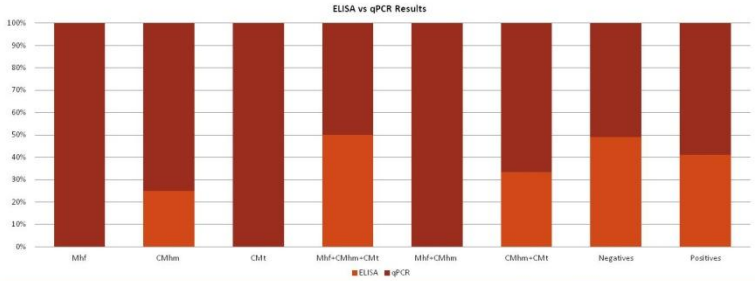
Material and Methods

Results

Positives	16
Negatives	78

Positives	23
Negatives	81

Positives	51
Negatives	53



Cytology	qPCR		Total
	Negative	Positive	
Negative	46	7	53
Positive	35	16	51
Total	81	23	104

Conclusions

Kappa values were calculated for qPCR – cytology (k=0,1836) and rELISA - qPCR (k=0,1093).
Sensitivity and specificity for rELISA was 25% and 85% respectively. For cytology the sensitivity was 69% whereas the specificity was 56%.
1 - Assuming the higher performance of the qPCR assay, a low agreement (k) was observed between the molecular data and the cytology, which was already expected.
2 - The results showed also a low convergence between rELISA and the qPCR, regardless of the feline mycoplasma species involved. Also, although this antigen seems to have some specificity (85%), the sensibility is low (25%). Therefore, considering all the data, it is concluded that this recombinant antigen may not be suitable for the detection of antibodies against feline mycoplasmas in naturally infected cats.

Funding acknowledgments: Funded by Project UID/CVT/276/2013 CIISA (CIISA- Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon)

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Appendix B – Table of results from *Mhf* DnaK antibody testing

Sample ID	Mean percentage of the positive control
704	1,9%
705	6,8%
706	0,0%
707	0,0%
708	0,0%
713	26,9%
714	0,0%
715	45,8%
717	5,6%
725	0,0%
726	0,0%
728	61,0%
747	0,0%
754	0,0%
762	0,0%
763	29,1%
764	18,8%
765	0,0%
770	5,3%
771	0,5%
772	3,3%
782	17,4%
783	0,0%
784	3,1%
786	77,8%
790	0,0%
791	10,6%
792	90,4%
793	0,8%
794	53,1%
795	0,0%
796	0,0%
797	0,0%
840	0,0%
841	0,0%
842	0,0%
843	0,0%
845	1,6%
880	0,0%
882	0,0%

Appendix B (cont.) – Table of results from *Mhf* DnaK antibody testing

Sample ID	Mean percentage of the positive control
883	0,0%
884	0,0%
885	0,0%
917	0,0%
945	0,0%
946	29,8%
947	0,0%
948	0,0%
949	0,0%
950	30,9%
951	0,0%
952	17,6%
954	0,0%
955	62,7%
971	0,0%
972	0,0%
973	0,0%
974	0,0%
976	0,0%
977	0,0%
980	0,0%
982	0,0%
983	8,6%
993	5,8%
1137	29,6%
1138	0,0%
1167	0,0%
1168	0,0%
1171	0,2%
1172	0,0%
1174	0,0%
1175	0,0%
1266	0,0%
1267	0,0%
1280	0,0%
1281	0,0%
1286	15,2%
1415	0,0%
1441	0,0%
1478	0,0%

Appendix B (cont.) – Table of results from *Mhf* DnaK antibody testing

Sample ID	Mean percentage of the positive control
1486	15,5%
1497	0,0%
1498	0,0%
1499	0,0%
1518	0,0%
1520	39,2%
1522	0,0%
1526	0,0%
1527	9,3%
1528	0,0%
1530	30,8%
1531	13,9%
1532	0,0%
1533	13,6%
1534	13,0%
1535	0,0%
1542	0,0%
1544	0,0%
1545	100,5%
1546	14,3%
1547	48,7%
1548	38,9%
1549	0,0%
1550	0,5%

Legend: Identificaton (ID)